



Osteoclasts regulation of Osteogenic Differentiation in Periodontal Ligament

Cells via RANK-RANKL Reverse Signaling

Sumit Suamphan

**A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor
of Philosophy in Oral Health Sciences**

Prince of Songkla University

2025

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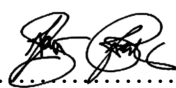
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ABSTRACT

Several *in vitro* studies have previously shown reverse signaling from osteoclasts that regulate osteoblast differentiation and mineralization. Extracellular vesicles (EVs) released from osteoclasts are key molecules. However, none of these studies reported the effect of this signaling on periodontal ligament (PDL) cells.

Objective: This study, therefore, aimed to investigate the osteogenic differentiation of human PDL cells as the result of interaction between the receptor activator of nuclear factor kappa B (RANK) on EVs released from mature human osteoclasts and membranous RANK ligand (RANKL) on human PDL cells.

Methods: Multinucleated mature human osteoclasts differentiated from peripheral blood mononuclear cells and human PDL cells were used in this study. The conditioned medium from mature osteoclasts (OC-CM), osteoclasts' extracellular vesicles (OC-EVs), or recombinant human RANK were used to induce osteogenic differentiation of the PDL cells. Osteogenic gene expressions, osteoblast differentiation, and biomineralization were investigated. The role of OC-CM and OC-EVs were validated by comparison with OC-CM from GW4869 treated osteoclasts (GW-OC-CM) or OC-EVs from GW4869 treated osteoclasts (GW-OC-EVs), respectively. Recombinant human RANKL or osteoprotegerin (OPG) were used for inhibition of RANK-RANKL reverse signaling.

Results: OC-CM and OC-EVs significantly upregulated osteogenic gene expressions, osteoblast differentiation, and biomineralization of the PDL cells ($p < 0.05$). GW-OC-CM and GW-OC-EVs significantly diminished osteogenic gene

expressions, osteoblast differentiation, and biomineralization of the PDL cells ($p < 0.05$). OPG and RANKL significantly reduced the osteogenic inductions in PDL cells ($p < 0.05$). However, no significant change was found from recombinant RANK treated PD cells.

Conclusion: Extracellular vesicles released from mature osteoclasts can induce osteogenesis of human PDL cells via RANK-RANKL reverse signaling.

Keywords : Extracellular vesicles, Osteoclast, Osteogenic differentiation, Periodontal ligament, RANKL

ชื่อวิทยานิพนธ์	การเปลี่ยนสภาพเป็นเซลล์สร้างกระดูกของเซลล์เอ็นดอทีคัลโดยการควบคุมของ เซลล์สลายกระดูกด้วยวิธีการส่งสัญญาณย้อนกลับแรงค้ำแรงค้ำไลแกน
ผู้เขียน	นายสุมิตร สุอำพัน
สาขาวิชา	วิทยาศาสตร์สุขภาพช่องปาก
ปีการศึกษา	2567

บทคัดย่อ

จากหลากหลายการศึกษาที่ผ่านมาได้แสดงให้เห็นถึงบทบาทที่แตกต่างออกไปจากเดิมของเซลล์สลายกระดูก ซึ่งเป็นการเหนี่ยวนำให้เซลล์สร้างกระดูกเกิดการแปรเปลี่ยน สะสมแร่ธาตุ และสร้างเนื้อเยื่อกระดูกขึ้นใหม่ ทั้งนี้โมเลกุลสำคัญในการส่งสัญญาณย้อนกลับจากเซลล์สลายกระดูกไปยังเซลล์สร้างกระดูก คือ เวซิเคิลนอกเซลล์ อย่างไรก็ตาม ยังไม่มีการรายงานถึงผลที่เกิดขึ้นในเซลล์เอ็นดอทีคัล

วัตถุประสงค์: เพื่อศึกษาถึงการแปรเปลี่ยนเป็นเซลล์สร้างกระดูกของเซลล์เอ็นดอทีคัลซึ่งเป็นผลจากปฏิสัมพันธ์ระหว่างแรงค้ำบนเวซิเคิลนอกเซลล์ที่หลังจากเซลล์สลายกระดูกเต็มวัยของมนุษย์ และแรงค้ำไลแกนบนผิวเซลล์เอ็นดอทีคัลมนุษย์

วิธีการศึกษา: เซลล์สลายกระดูกที่โตเต็มวัยของมนุษย์ซึ่งได้มาจากการเหนี่ยวนำเซลล์โมโนไซต์จากเลือดของอาสาสมัคร ถูกนำมาเลี้ยงในห้องปฏิบัติการเพื่อเก็บอาหารที่ใช้เลี้ยงเซลล์สลายกระดูกมาแล้วระยะหนึ่งไปทำให้เข้มข้นขึ้น หรือ นำไปแยกเวซิเคิลนอกเซลล์

เซลล์เอ็นดอทีคัลถูกเหนี่ยวนำด้วยอาหารเลี้ยงเซลล์ที่ถูกทำให้เข้มข้น, เวซิเคิลนอกเซลล์, หรือโปรตีนแรงค้ำ เพื่อดูการแปรเปลี่ยนเป็นเซลล์สร้างกระดูก

ทำการยืนยันบทบาทของเวซิเคิล นอกเซลล์ ด้วยการศึกษาเปรียบเทียบกับอาหารที่ใช้เลี้ยงเซลล์มาแล้วระยะหนึ่งไปทำให้เข้มข้นขึ้น หรือ เวซิเคิลนอกเซลล์ ที่ได้มาจากเซลล์สลายกระดูกที่ได้รับสารยับยั้งการหลั่งเวซิเคิลนอกเซลล์

การส่งสัญญาณย้อนกลับแรงค์-แรงค์ไลแกน ถูกยับยั้งด้วยโปรตีนแรงค์ไลแกนหรือออสทีโอโปรทีเจริน ตามลำดับ

ผลการศึกษา: อาหารที่ใช้เลี้ยงเซลล์มาแล้วระยะหนึ่งไปทำให้เข้มข้นขึ้น และ เวชikelนอกเซลล์ที่ได้มาจากเซลล์สลายกระดูก ส่งผลให้เซลล์เอ็นดอทีลียัลเพิ่มระดับการแสดงออกของยีนส์ที่เกี่ยวข้องกับการแปรเปลี่ยนเป็นเซลล์สร้างกระดูก รวมถึงเพิ่มการสะสมแร่ธาตุอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเหนี่ยวนำด้วยอาหารที่ใช้เลี้ยงเซลล์มาแล้วระยะหนึ่งไปทำให้เข้มข้นขึ้น หรือ เวชikel นอกเซลล์ ที่ได้จากเซลล์สลายกระดูกที่ได้รับสารยับยั้งการหลั่งเวชikelนอกเซลล์ ไม่พบการเปลี่ยนแปลงระดับการแสดงออกของยีนส์และการสะสมแร่ธาตุอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) การยับยั้งแรงค์ไลแกนบนผิวเซลล์เอ็นดอทีลียัล หรือการยับยั้งแรงค์บนผิวเวชikel ก็ไม่พบการเปลี่ยนแปลงระดับการแสดงออกของยีนส์และการสะสมแร่ธาตุอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เช่นเดียวกัน แต่การเหนี่ยวนำเซลล์เอ็นดอทีลียัลด้วยโปรตีนแรงค์กลับไม่พบการเปลี่ยนแปลงอย่างมีนัยสำคัญของการแสดงออกของยีนส์ และการสะสมแร่ธาตุ

สรุปผลการศึกษา : เวชikelนอกเซลล์ที่หลังจากเซลล์สลายกระดูกสามารถเหนี่ยวนำให้เซลล์เอ็นดอทีลียัลแปรเปลี่ยนเป็นเซลล์สร้างกระดูก ผ่านวิธีการส่งสัญญาณย้อนกลับของแรงค์-แรงค์ไลแกน

คำสำคัญ : การแปรเปลี่ยนเป็นเซลล์สร้างกระดูก, เซลล์สลายกระดูก, เวชikelนอกเซลล์, เอ็นดอทีลียัล, แรงค์ไลแกน

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had failed another chance. But no, it turned out that all this time, she continued to believe in me. And when the right moment came, she offered me another opportunity, even though I had long given up on believing that I could do it. But not for her. And so, I decided to pursue my PhD again, following her advice. This led me to constantly trouble her with various matters over the past four years. In the end, I can only say one thing: “Because she believed in me, I was able to do it.”

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Sumit Suamphan

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LIST OF ABBREVIATIONS AND SYMBOLS

ALP	alkaline phosphatase
BSP2	bone sialoprotein 2
cDNA	complementary deoxyribonucleic acid
c-Fms	colony-stimulating factor-1 receptor
CM	conditioned medium
COL1A1, Colia1	collagen type 1 alpha 1 chain
DC-STAMP	dendritic cell-specific transmembrane protein
DLS	dynamic light scattering
DMEM	Dulbecco Modified Eagle Medium
DMSO	dimethyl sulfoxide
ESCRT	endosomal sorting complex required for transport
EVs	extracellular vesicles
FBS	fetal bovine serum
GDNF	glial cell-derived neurotrophic factor
GW-OC-CM	GW4869-treated mature osteoclasts
GW-OC-EVs	OC-EVs from GW4869 treated osteoclasts
ILVs	intraluminal vesicles
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
miRNA	micro ribonucleic acid
MC	monocyte

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUE)

MSC	mesenchymal stem cell
MVBs	multivesicular bodies
Mø	macrophage
NFATc1	nuclear factor of activated T cells 1
NTA	nanoparticle tracking analysis
OC-EVs	Osteoclasts' extracellular vesicles
OC-CM	conditioned medium from mature osteoclasts
OCN	osteocalcin
OPN	osteopontin
OSM	oncostatin M
OSX	osterix
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
RUNX2, Runx2	runt-related transcription factor 2
TRAcP	tartrate-resistant acid phosphatase
TRAFs	TNF receptor-associated factors
TSG101	tumor susceptibility gene 101
TEM	transmission electron microscope
PE	phycoerythrin

CHAPTER 1

INTRODUCTION

Background and rationale of the study

In bone biology, receptor activator of nuclear factor kappa B (RANK) and its ligand (RANKL) are major regulatory molecules in bone resorption. RANKL directly binds to membrane-bound RANK on osteoclast lineage cells for osteoclast differentiation, maturation and function.⁽¹⁾ Two forms of RANKL, soluble RANKL and membrane-bound RANKL are produced by bone cells and both stimulate osteoclast formation *in vitro*.⁽²⁾

Emerging evidences indicate that osteoclasts provide coupling signals for not only play a role in bone resorption but also co-ordinate in bone formation by releasing the regulatory molecule known as extracellular vesicles (EVs) for regulation of osteoblast function.⁽³⁻⁶⁾ EVs are small membrane-enclosed bodies shed from the cell surface with engulfed cytoplasmic contents including cell-surface proteins, functional proteins, and genetic materials.⁽⁷⁾ RANK was identified on the membrane of EVs released from mature osteoclasts and demonstrated the ability to promote bone formation by triggering mRANKL of osteoblasts along the activation of runt-related transcription factor 2 (*RUNX2*) through PI3K/mTORC1 signaling pathway. This mechanism called RANK-RANKL reverse signaling which are associated in coupling signals of bone in both resorption and formation activities.⁽³⁾

The periodontal ligament (PDL) is a thin layer of connective tissue lied between cementum and bone socket. Mineral homeostasis in periodontal space is an important process regulated by surrounding cells and molecules for stabilization of physiological

state in PDL tissue for any change in environmental equilibrium.⁽⁸⁾ Many studies indicated that the cells from PDL perform many characteristics of bone cells, including the mineralization ability, expression of the osteogenic markers and also the potential to differentiate into functional osteoblast-like cells of PDL cells has been validated.⁽⁹⁻¹¹⁾

In bone remodeling process, regulatory mechanisms of osteoclastogenesis by RANKL from PDL cells have been widely studied through cell-to-cell contact and paracrine manner which described the function of RANK on osteoclast membrane.⁽¹²⁻¹⁵⁾ Anyway, the role of osteoclasts in regulation of PDL cells has never been reported. Aside from the RANKL reverse signaling of EV-derived osteoclasts and osteoblasts,^(3, 5) the possibility of osteoclast function in associated with osteogenic activities of PDL cells is hypothesized. A new theory of orthodontic tooth movement called “Biphasic theory” was introduced in 2018 by Alikhani, *et al*⁽¹⁶⁾ which breakthrough classical theories about cell involved in the location of pressure and tension sites independently. This theory illustrated new prospective of osteoclast role from histological sections of root at initial time of tooth movement and showed the appearance of osteoclasts in both pressure and tension sites. Anyway, there is no particular explanation for the role of osteoclasts in tension sites. The finding of this study will be undertaken to find out the role of osteoclasts on PDL cells for the first time and to provide some description to this issue of controversy in orthodontic tooth movement. Furthermore, it can be expected in future clinical researches by delivering EVs as biological nanoparticles which may have superior characteristic in biocompatibility.

In this proposed study, culture of PDL cells with EVs derived mature osteoclasts will be established to test whether the present of osteoclasts can regulate osteogenic differentiation of PDL cells via RANKL reverse signaling.

Objectives

To study the effect of extracellular vesicles released from mature osteoclasts on osteogenic differentiation in PDL cells through the RANK-RANKL reverse signaling.

CHAPTER 2

REVIEW OF LITERATURE

Biology of tooth movement

The biology of orthodontic tooth movement, despite being studied for decades, continues to be a key area of research. Advances in technology have provided valuable insights into the molecular, cellular, and tissue-level reactions to orthodontic forces. Understanding these mechanisms is crucial, as it forms the basis of orthodontics, which relies on moving teeth through the alveolar bone. While the biological processes involved in tooth movement are well-established, optimizing the speed of this movement and minimizing potential risks remain ongoing challenges for researchers and clinicians. Although the tissue responses that allow orthodontic tooth movement are understood, the underlying mechanisms driving these reactions are not fully known. For instance, one important question that remains unanswered is whether the PDL plays a role in regulating the rate of tooth movement.

The alveolar bone is considered one of the most responsive skeletal tissues in the body. When an orthodontic force is exerted on a tooth, a series of coordinated signals are transmitted from the tooth through the PDL to the alveolar bone. The key bone cells involved in enabling tooth movement include osteoblasts, which form new bone; osteoclasts, which break down bone; and osteocytes, which sense mechanical changes.

Orthodontic forces create stresses that are transmitted through the PDL to the alveolar bone, leading to tooth movement. According to traditional theories, the biology behind tooth movement is based on three key principles:

a) Cellular involvement: Compression triggers osteoclastogenesis and activates osteoclast function, while tension stimulates osteoblasts. As a result, osteoclasts are found at compression sites, and osteoblasts are located at tension sites.

b) Location: The catabolic and anabolic responses occur independently within the PDL, on opposite sides of the tooth.

c) Timing: Despite being independent, the catabolic and anabolic phases happen simultaneously, as both compression and tension are present at the same time.

This classical theory of tooth movement suggests that the primary target of orthodontic forces is the PDL. Different types of orthodontic forces create specific compression and tension patterns within the PDL, with the duration of these forces playing a role in their effects. When a compressive force is sustained, gingival crevicular fluids are pushed out of the PDL space, facilitating tooth movement and further compressing the PDL. The immediate consequence of this compression is the constriction of blood vessels, leading to reduced blood flow, oxygen, and nutrient supply (hypoxia) at the compression site. Depending on the level of pressure and blood flow disruption, some cells may undergo apoptosis, while others may die in a nonspecific manner, resulting in necrosis, which is histologically identified as the cell-free zone. It is important to note that apoptotic or necrotic changes are not limited to PDL cells, as osteoblasts and osteocytes in the surrounding alveolar bone may also experience cell death due to orthodontic forces. However, triggering bone cell function via inflammatory cytokines and mediators has not been fully explained in the theory.

Research suggests that orthodontic forces are converted into a sequence of catabolic processes followed by anabolic activity in the alveolar bone. Based on these

findings, Alikani *et al.* introduced the Biphasic Theory of Tooth Movement to explain the pattern of bone remodeling during tooth movement.⁽¹⁶⁾

The Biphasic Theory proposes that orthodontic tooth movement occurs through two successive phases of alveolar bone remodeling triggered by orthodontic force. The Catabolic Phase occurs first, followed by the Anabolic Phase, with specific cellular and molecular events defining the boundaries of each phase. Both compression and tension cause damage to the PDL, triggering an aseptic inflammatory response that leads to the formation of an area of osteoclastogenesis. The tooth then moves into the space created by the osteoclasts, while the osteoclastogenesis area gradually shifts in the direction of the applied force. As the Catabolic Phase comes to an end, the Anabolic Phase begins, with osteoblasts forming a perimeter of osteogenesis. The osteoclastogenesis perimeter is essential for activating the osteogenic perimeter. It is important to note that, when examining the histological evidence for the Biphasic Theory, it may seem as though these two phases occur independently.

Periodontal ligament cells

Stem cells have attracted considerable interest in tissue engineering due to their powerful regenerative abilities and their capacity to modulate immune responses. Dental stem cells, a type of mesenchymal stem cells, are known for their exceptional potential to differentiate into various cell types, such as odontoblasts and osteoblasts, under specific conditions. The application of these mesenchymal stem cells in their natural environment presents a promising method for addressing chronic dysbiosis. Furthermore, periodontal regenerative therapy using dental stem cells has emerged as a reframing approach, with the potential to restore the functionality of the periodontal

ligament. Stem cells extracted from the PDL were initially isolated by Seo *et al* in 2005,⁽¹⁷⁾ can be cultured in a xeno-free medium while maintaining their morphological characteristics and markers linked to pluripotency. The PDL is primarily composed of fibers, cells, and the surrounding extracellular matrix, with blood vessels and nerve bundles providing nourishment. Collagen fibrils make up a significant portion of the PDL fibers, accounting for approximately 50–75% of its volume. The majority of these fibrils are type I collagen, with smaller amounts of type III, IV, V, VI, and XII collagen. These collagen fibers are organized into fiber bundles, with the principal fibers playing a key role in anchoring the tooth by attaching to the cementum on one side and the alveolar bone on the other. Moreover, the principal fibers also contain elastic fibers, such as oxytalan, which contribute to the formation of the neural and vascular network within the PDL and help regulate vascular flow.

The primary and most functionally important cells of the PDL are fibroblasts, also known as PDL cells. These cells are crucial for synthesizing collagen fibers and play a key role in the ongoing remodeling and regeneration processes within the PDL. Both the fibroblasts and their contributions are vital for the regeneration of the periodontal ligament. The structural stability of the PDL is maintained through the rapid turnover of collagen, with collagen fibers having a relatively short lifespan of just a few days. This ongoing process is largely driven by the activity of PDL cells, which serve as a self-renewing system to maintain tissue balance. PDL cells generate new collagen fibers through cell proliferation, compensating for the high rate of collagen degradation. During this process, PDL cells efficiently eliminate aged or damaged collagen fibers and replace them with newly formed ones. However, this regenerative ability is significantly reduced in the presence of inflammation.

For functional regeneration of the PDL, the newly formed PDL fibers must not only regenerate collagen but also reattach to the alveolar bone and cementum, forming Sharpey's fibers. Under normal physiological conditions, remodeling of the PDL on the alveolar bone side occurs more rapidly than on the cementum side, possibly due to the greater maturity of the collagen on the cementum surface. This difference may be linked to the faster turnover rate of alveolar bone. Consequently, promoting the reconstruction of alveolar bone is essential for the proper formation of Sharpey's fibers. ⁽¹⁸⁾ The phenotypic consistency of cell populations throughout the PDL tissue may play a role in maintaining the integrity of different areas of the PDL. In fact, staining for various phenotypic markers reveals relatively uniform expression in both the root and bone-associated regions of the PDL tissue. ⁽¹⁹⁾

Despite the continuous physiological movement of teeth and the presence of cells with osteoblastic markers in the periodontal ligament, the resident cells in the ligament consistently produce regulatory factors that maintain its width by inhibiting osteogenesis. ⁽²⁰⁻²²⁾ The impact of various signaling pathways on the osteogenic differentiation of PDL cells demonstrates that pathways such as Wnt, NF- κ B, MAPK, BMP, and PI3K/AKT play crucial roles in this process. These pathways operate through distinct transduction mechanisms but also interact with one another, collectively regulating osteogenic differentiation. ⁽²³⁾

Osteogenic differentiation

The mesenchymal stem cell differentiation regarding the osteogenic lineage is regulated by various molecules. The initiation of osteogenic differentiation appears when the master of osteogenic transcriptional factors, such as *RUNX2* and Osterix

(*OSX*) are activated following by the transcription of early osteogenic genes, such as alkaline phosphatase (*ALP*) and collagen type 1 alpha 1 chain (*COL1A1*) accompany with other osteogenic proteins, such as bone sialoprotein 2 (*BSP2*), osteopontin (*OPN*), and osteocalcin (*OCN*).

Runx-related transcription factor 2 (*Runx2*) belongs to The Runx family which composed of *Runx1*, *Runx2*, and *Runx3* gene. *Runx2* is necessary for engagement of mesenchymal cells into osteoblast lineage. *Runx2* as a key molecule in osteoblast differentiation induces expression of osteoblast marker gene including *Col1a1*, *Col1a2*, *BSP*, *OPN*, and *OCN*.^(24, 25) During uncommitted mesenchymal cell stage, the expression of *Runx2* is low. Upregulation of *Runx2* is found in pre-osteoblasts and highest in the stage of immature osteoblasts.⁽²⁵⁾ Then β -catenin and *OSX* conduct pre-osteoblast differentiation to immature osteoblasts. In mature osteoblasts, the level of expression is declined. Overexpression of *Runx2* in transgenic mice showed that *Runx2* inhibited osteoblast differentiation at a late stage and suggested that *Runx2* may has a role in reservation of immature osteoblasts.⁽²⁶⁾

OCN is one of the non-collagenous proteins that most abundant in bone tissue. As a biomarker, *OCN* is used in the present of mature osteoblasts. In bone remodeling, *OCN* exerts role in many aspects including inhibition of osteoblast differentiation, inducing migration and adhesion of osteoclasts.⁽²⁷⁾ The expression of *OCN* is mainly controlled by *Runx2* protein.⁽²⁵⁾ Although both the *OCN* promoter and *Col1a1* promoter drive transgene expression in osteoblasts, the *OCN* promoter is active in more mature osteoblasts.⁽²⁸⁾

Micro ribonucleic acids (miRNAs) are a group of noncoding, single-stranded RNA molecules, typically 19–24 nucleotides in length, that are produced by

endogenous genes. These miRNAs attach to the 3' UTR or coding region of target mRNA through complementary base pairing. This binding recruits the RNA-induced silencing complex, which either leads to the degradation of the mRNA or inhibits its translation into protein. miRNAs derived from osteoclasts within EVs may play a role in both bone resorption and formation, potentially acting as "coupling factors associated with EVs." Deep sequence analysis showed 70 common miRNAs present in EVs from RAW 264.7 cells.⁽²⁹⁾ One such miRNA, miR-214-3p, has been identified in EVs originating from osteoclasts. The level of supernatant EV miR-214-3p in mature osteoclasts was higher than osteoclast precursors.⁽³⁰⁾ These miR-214-3p-containing EVs have been shown to affect pre-osteoblastic cells in a paracrine manner, suppressing bone formation by directly interacting with and inhibiting the Activating transcription factor 4 (*Atf4*) mRNA. This suggests that miR-214 in EVs could be involved in the communication between osteoclasts and osteoblasts.⁽³¹⁾ Moreover, osteoclast-derived miR-23a-5p-containing EVs were observably increased in EVs from RANKL-induced RAW 264.7 cells relative to RAW 264.7 cells.⁽³⁰⁾ EVs derived from RANKL-induced RAW 264.7 cells contain miR-23a-5p could target *Runx2* and affect osteogenesis via downregulation of *Runx2* to inhibit osteogenic differentiation.⁽⁶⁾

Osteogenic differentiation of human periodontal ligament cells

PDL cells are ability to differentiate into osteogenic lineage and have many osteoblast-like properties. In 1886, Kawase, *et al* showed that PDL cells exhibited alkaline phosphatase activity which suggested that PDL tissue could be the origin of osteoprogenitor cells. *In vivo* study of Roberts and Weiss in 1974 demonstrated the maturation scheme of bone-forming cells from fibroblastic precursor cells in rat PDL.

⁽¹¹⁾ The biological characteristics of cells within the PDL have been studied extensively, especially in their osteogenic potential. The osteoblast characteristics of PDL cells are regularly identified *in vitro* via expression of osteogenic markers, response to bone-inductive factors and capacity to form mineralize nodule. Many osteogenic marker genes of PDL cells such as *ALP*, *Col1a1*, *Runx2*, *OSX*, *OCN*, *OPN*, *BSP*, Dentin sialophosphoprotein, core binding factor- α 1, *BMP2*, *BMP4* and *BMP7* were reported. ^(10, 32, 33) Osteoblastic lineage cells can promote osteogenic differentiation of PDL cells which were shown in co-culture model and EV-derived induction. ⁽³⁴⁻³⁶⁾ Expression of most osteogenic genes in PDL cells can be early detected after 3-7 days cultured with osteogenic induction medium. ⁽³³⁾ The level of mRNA expression of most bone-inductive factors can be analyzed up to 14 days after osteogenic induction. ^(9, 10, 37)

Beside RANK-RANKL, interaction between ephrin2 and ephB4 does involve in bone remodeling in bidirectional regulation. Forward signaling from ephrin2 on osteoclast stimulates osteoblast differentiation and induces bone formation. Reverse signaling from ephB4 on osteoblast inhibits osteoclast function. ⁽³⁸⁾ Bidirectional ephrin2-ephB4 signaling also has a role in osteogenic differentiation of PDL cells. ⁽³⁹⁾ Within co-culture, signal crosstalk between pre-osteoblasts and PDL cells resulted in osteogenic differentiation of PDL cells. ⁽⁴⁰⁾ However, the role of ephB4 from osteoclasts on PDL cells has not been studied. PDL cells are capable of selection and attraction to osteoclast precursors following the retraction and migration of osteoclast precursors toward bone surface area. Subsequently, proliferation and fusion of precursors occurs and then osteoclast accomplishment. The RANKL-RANK axis takes a key role in this process. Expression of sRANKL and mRANKL via PDL cells has been demonstrated in several studies. ^(12, 13, 41, 42) The TNFSF11 gene, which encodes RANKL, can be found

in numerous cell types and also PDL cells.⁽⁴³⁻⁴⁵⁾ PDL cells under dexamethasone and $1\alpha,25$ -dihydroxyvitamin D3 exhibited a declination in OPG secretion and elevation in the levels of RANKL and *TNFSF11* mRNA.⁽⁴³⁾ The recent study showed that suppression the expression of *TNFSF11* with Artemisinin (osteoarthritis drug) can inhibited the PI3K–AKT–mTOR pathway.⁽⁴⁶⁾

Recent studies have shown that miRNAs play a role in regulating the osteogenic differentiation of PDL cells through various signaling pathways. Overexpression of miRNA-132 could enhance the osteogenic differentiation of PDL cells by activating the Wnt/ β -catenin signaling pathway.⁽⁴⁷⁾ miR-584-5p negatively regulates the osteogenic differentiation of PDL cells.⁽⁴⁸⁾ miRNA-222 could decrease the expression of Smad2 and Smad7, thereby inhibiting the osteogenic differentiation of PDL cells.⁽⁴⁹⁾ These findings highlight the significant impact of the interaction between miRNAs and signaling pathways on the osteogenic differentiation of PDL cells, offering new insights for potential strategies to regulate this process.

Osteoclasts

Osteoclasts are multinucleated cells with the ability to degrade bone tissue by secreting H^+ , Cl^- , cathepsin K, and matrix metalloproteinases (MMPs). Osteoclast precursors develop from monocyte/macrophage lineage cells, which originate from myeloid progenitors. Monocytes fuse together to form multinucleated cells, which then become osteoclasts. Mature osteoclasts are identified as multinucleated cells with more than three nuclei or up to 100 nuclei. Osteoclasts grow by fusion or increased transcriptional output, which enhances genome content. Osteoclasts with larger cell sizes are more functional in a stimulating environment.^(50, 51)

Macrophage colony-stimulating factor (M-CSF) binding to the colony-stimulating factor-1 receptor (c-Fms) in osteoclast precursors supports cell survival and induces the expression of RANK. The downstream signaling of RANK from RANKL stimulation leads to osteoclastogenesis. RANKL-RANK signaling activates osteoclastogenic transcription factors, including NF κ B, activator protein 1, and nuclear factor of activated T cells 1 (NFATc1). NFATc1 is crucial for triggering the expression of genes required for the differentiation of progenitor cells into preosteoclasts. Dendritic cell-specific transmembrane protein (DC-STAMP) is involved in preosteoclast differentiation and plays an important role in the fusion of osteoclast mononuclear cells. Cathepsin K is a lysosomal cysteine protease predominantly released from mature osteoclasts, where it breaks down collagen and matrix proteins during bone resorption. Tartrate-resistant acid phosphatase (TRAcP) positivity in multinucleated cells is considered a crucial cytochemical marker for osteoclasts.^(52, 53)

Several studies have demonstrated the role of the osteoclast lineage in bone formation. The osteogenic differentiation of mesenchymal stem cell (MSC) lineages was induced by conditioned medium (CM), EVs, and apoptotic bodies derived from osteoclast lineage cells, as shown in Table 1.

Table 1 List of studies on osteogenic differentiation of mesenchymal stem cell lineages induced by conditioned medium or extracellular vesicles from osteoclast lineage

Study	Source of CM or EVs	Target cells	Signaling molecule/pathway
Xian-Min Liao 2022 ⁽⁵⁴⁾	macrophages (THP-1 cells) EVs	human PDL cells	differentially expressed miRNAs
Zhang 2022 (55)	osteoclasts (RAW264.7 & primary human cells) CM	mouse MSCs human MSCs	-
Liang 2021 ⁽⁵⁶⁾	osteoclasts (primary mouse cells) CM, GW-CM, EVs	mouse MSCs	miR-324
Stessuk 2021 (57)	macrophages (primary human cells) CM	human MSCs	-
Yi 2020 ⁽⁵⁸⁾	osteoclasts (primary mouse cells) CM	mouse MSCs	glial cell-derived neurotrophic factor (GDNF)
Ma 2019 ⁽⁵⁹⁾	osteoclasts (RAW264.7) apoptotic bodies	MC3T3-E1	PI3K–Akt–mTOR–S6K

Table 1 List of studies on osteogenic differentiation of mesenchymal stem cell lineages induced by conditioned medium or extracellular vesicles from osteoclast lineage (continue)

Study	Source of CM or EVs	Target cells	Signaling molecule/pathway
Almeida 2019 (60)	osteoclasts (primary human cells) CM	human MSCs	-
Ikebuchi 2018 (3)	osteoclasts (RAW264.7 & primary mouse cells) GW-EVs, EVs	MC3T3-E1 & mouse <i>in vivo</i>	RANK-RANKL & mTORC1
Zhang 2017 (61)	recombinant mouse RANK	MC3T3-E1	RANK-RANKL MAPK p38
Matsuoka 2014 ⁽⁶²⁾	osteoclasts (primary mouse cells) CM	mice stromal cells	C3- C3a receptor

Table 1 List of studies on osteogenic differentiation of mesenchymal stem cell lineages induced by conditioned medium or extracellular vesicles from osteoclast lineage (continue)

Study	Source of CM or EVs	Target cells	Signaling molecule/pathway
Ekstrom 2013 (⁶³)	LPS induced-monocytes (primary human cells) EVs	human MSCs	-
Fernandes 2013 (⁶⁴)	macrophages & osteoclasts (primary human cells) CM	adipose tissue- derived MSCs	Oncostatin M canonical Wnt
Chen 2012 (⁶⁵)	osteoclasts (RAW264.7) CM	MC3T3-E1	-
Kreja 2010 (⁶⁶)	osteoclasts (primary human cells) CM	human MSCs	-
Omar 2011 (⁶⁷)	LPS induced-monocytes (primary human cells) CM	bone marrow- derived MSCs	-
Pederson 2008 (⁶⁸)	Osteoclasts (RAW 264.7 & human cell line) CM	human MSCs	Wnt/BMP sphingosine-1-phosphate

RANK-RANKL-osteoprotegerin (OPG) complex

The identification of the RANK/RANKL/OPG system in the mid-1990s, which plays a key role in regulating bone resorption, marked a significant advancement in our understanding of bone modeling and remodeling processes. Prior to this discovery, it was known that osteoblastic stromal cells influenced osteoclast formation, but it was unexpected that they did so through the expression of TNF superfamily members such as RANKL and OPG. This signaling pathway, involving the RANK receptor, was also found to have broader functions beyond regulating bone remodeling. The RANKL/RANK pathway controls osteoclast formation, activation, and survival in both normal bone processes and pathological conditions characterized by elevated bone turnover. Additionally, OPG protects bone from excessive resorption by binding to RANKL, thereby preventing its interaction with the RANK receptor.⁽⁶⁹⁾

OPG is produced by various cell types beyond osteoblasts. Most of the factors that stimulate RANKL expression in osteoblasts also influence OPG production. While some conflicting findings exist, the general pattern is that when RANKL expression increases, OPG expression is either downregulated or does not increase to the same extent, resulting in a shift in the RANKL/OPG ratio that favors osteoclastogenesis. Changes in the RANKL/OPG ratio, whether through an increase in RANKL, a decrease in OPG, or a combination of both, can lead to an increase in osteoclast numbers and activity.^(70, 71)

It has become increasingly evident that osteoclasts do more than just resorb bone; they also play a role in regulating osteoblast activity, both positively and negatively. Additionally, osteoclasts are involved in the process of hematopoietic stem cells exiting the bone marrow and entering the bloodstream, and they function as

immunomodulators in various pathological conditions.^(38, 72-74) A crucial initial step in the signaling cascade following RANKL binding to RANK involves the attachment of TNF receptor-associated factors (TRAFs) to specific regions of RANK's cytoplasmic domain. As a transmembrane protein, RANK, like other TNF family receptors, lacks intrinsic protein kinase activity to directly mediate signaling. TRAFs 2, 5, and 6 all bind to RANK, but only TRAF6 appears to play a vital role in osteoclast precursors and osteoclasts, as the deletion of TRAF6 alone leads to osteopetrosis. Osteopetrosis was observed in TRAF6-deficient mice generated by two separate research teams. Interestingly, one group of mice exhibited normal osteoclast numbers, though the cells were inactive, while the other group had no osteoclasts at all. The reason for these two distinct osteoclast phenotypes in the absence of TRAF6 remains unclear. Several signaling pathways are activated through RANK/TRAF-mediated protein kinase signaling, with four directly promoting osteoclast formation (IKK/NF- κ B, JNK/AP-1, c-myc, and calcineurin/NFATc1), and three facilitating osteoclast activation (Src, MKK6/p38/MITF) and survival (Src, ERK).⁽⁷⁵⁻⁷⁸⁾

RANK-RANKL reverse signaling

When RANKL signaling is triggered in osteoblasts, these cells produce various growth factors and their receptors, which stimulate differentiation in an autocrine fashion. These factors and receptors, generated through RANKL reverse signaling, can then sequentially influence nearby cells to further promote differentiation in a paracrine manner. For direct cell-to-cell contact, forward signaling of the RANKL-RANK pathway is activated to drive osteoclastogenesis, as shown in co-culture studies between osteoclasts and PDL cells.^(12, 79) Both the reverse signal from RANK on osteoclasts to

RANKL on osteoblasts and the forward signal from RANKL to RANK are thought to play crucial roles in the coupling of bone formation and resorption. Recent research by Ikebuchi *et al* ⁽³⁾ supports this hypothesis, suggesting that extracellular vesicles containing RANK secreted by osteoclasts regulate osteoblast differentiation. Modulators of RANKL reverse signaling, such as WP9QY (a peptide designed to resemble one of the cysteine-rich domains of TNF receptor type I, known for binding to TNF α and inhibiting its activity) and RANKL agonist antibodies, show promise as potential treatments for metabolic bone diseases. WP9QY has been shown to accelerate BMP-2-induced calvarial bone regeneration, stimulate osteoblast differentiation in mice, and restore alveolar bone loss by inhibiting osteoclastogenesis and promoting osteoblast differentiation in OPG-KO mice. ⁽⁸⁰⁾ A bifunctional RANKL antibody that inhibits osteoclastogenesis and activates RANKL reverse signaling also prevented bone loss in ovariectomized mice. ⁽³⁾

RANK expression in osteoclasts

The study of Ikebuchi *et al* showed that RANK was detected in osteoclast precursors incubated with the ectodomain of RANKL fused to sRANKL for 60-120 hours, but not in those incubated for 0-48 hours. This indicates that RANK is expressed in mature osteoclasts and is negligible in preosteoclasts. ⁽³⁾ The study also demonstrated that RANKL-induced expression of RANK depends on JNK1/2 signaling, compared to other MAP kinases. ⁽⁸¹⁾ Additionally, apart from RANKL, RANK levels can be increased in osteoclast precursors after TNF α treatment, both *in vitro* and *in vivo*. ⁽⁸²⁾

Extracellular Vesicles

EVs have been observed in a variety of body fluids, e.g., blood, saliva, and cerebrospinal fluid.⁽⁸³⁾ EVs are secreted by all cells in both health and disease.⁽⁸⁴⁾ EVs may be classified according to size, density, or surface antigen. The significance of EV studies lies in their ability to trigger target cells. Variation in EV surface components apparently affects their recognition and capture by acceptor cells. EVs can transmit information to acceptor cells by acting at the cell surface. Evidence shows that there is considerable overlap in the diameter and components of exosomes and microvesicles, aside from their secretion mechanisms. Due to this overlap in characteristics, EV subtypes may not be completely separable. For this reason, the International Society for Extracellular Vesicles recommends using the generic term “extracellular vesicles” to define all lipid bilayer-enclosed extracellular structures.⁽⁸⁵⁾

The biogenesis of EVs generally occurs within the endocytic pathway through the inward budding of the endosomal membrane, which results in the formation of very small vesicular structures contained within the endosome lumen, called intraluminal vesicles (ILVs). ILVs represent pre-secreted exosomes that may be expelled into the pericellular space upon the fusion of endosomes in the late stage, called multivesicular bodies (MVBs). The diameter of EVs is approximately 0.05 to 0.15 μm .⁽⁸⁶⁾ Budding away from the cytosol is a crucial property shared by EVs, distinguishing them from other intracellular budding vesicles. EV membranes are lipid bilayers with integrated proteins that enclose the contents inside, protecting them from nucleases and proteases.⁽⁸⁷⁾

All EVs from diverse cell types carry a common set of proteins, such as transmembrane proteins from the tetraspanin family (CD9, CD63, and CD81),

programmed cell death 6-interacting proteins, major histocompatibility complex (MHC) class II molecules, and tumor susceptibility gene 101 (TSG101) protein. TSG101 is a member of the endosomal sorting complex required for transport (ESCRT) proteins, which associate in successive complexes at the membrane of EVs to regulate cargo targeting inside. The presence of TSG101 and other ESCRT proteins or ESCRT-accessory molecules in EV preparations is often considered proof of their origin.⁽⁸⁸⁾ EVs released from the same type of cells may differ in the expression of cell surface receptors and their contents, depending on whether the cells are stimulated or exposed to certain environmental conditions, with mechanical stimuli being one such factor.^(34, 89)

EVs can communicate with recipient cells by interacting with the cell surface, without transferring their intra-vesicular contents. Variations in the size and surface characteristics of EVs likely affect how they are recognized and internalized by target cells. However, the mechanisms that occur after different subtypes are internalized by recipient cells remain unclear.⁽⁸⁷⁾

The regulatory mechanisms governing EV uptake by recipient cells remain uncertain. This process may depend on the metabolic status of the recipient cell, which influences the uptake of extracellular molecules and vesicles. Once inside the cells, internalized EVs can be degraded by lysosomes. EVs may also merge with existing endosomes, releasing their contents into the cytoplasm of recipient cells.⁽⁹⁰⁾ Following their binding to recipient cells, EVs can either remain attached to the plasma membrane, detach, directly merge with the membrane, or be internalized through various endocytic mechanisms.⁽⁹¹⁾

RANK- containing extracellular vesicles

RANK is a transmembrane protein present in EVs, allowing them to act at a distance from their cell of origin.⁽⁹²⁾ Mass spectrometry revealed the presence of several proteins with varying levels of expression, identifying RANK as notably more abundant in EVs derived from osteoclasts compared to those from precursor cells.⁽⁹³⁾ EVs containing RANK have been recognized as important mediators of intercellular signaling in bone. The first identification of RANK in EVs was demonstrated in a study comparing EVs released from preosteoclasts and mature osteoclasts. This early finding of RANK-EVs has since been validated by many subsequent studies.^(56, 89, 94) RANK in EVs is more highly expressed at the later stages of osteoclast differentiation.^(93, 95) Immunofluorescent staining revealed that RANK was concentrated in EVs released by mature osteoclasts.⁽³⁾ The size of the EVs in which RANK was detected was approximately 0.05 μm in diameter, which is consistent with RANK-containing EVs being exosomes. It has been suggested that RANK-EVs might act as antagonists to the RANKL-RANK interaction, similar to OPG. On the other hand, OPG may be capable of binding to RANKL, competing with RANK-EVs.⁽⁹³⁾

RANK-EVs act as coupling factors that promote bone formation to replace bone resorbed by osteoclasts. Unlike OPG, RANK-EVs from mature osteoclasts activate the RANKL reverse signaling pathway in osteoblasts, even when these osteoblasts are not in direct contact with osteoclasts.⁽⁹⁶⁾ The RANKL reverse signaling pathway, which is stimulated to trigger osteoclast differentiation, cannot stimulate RANKL reverse signaling in osteoblasts through direct interactions.⁽³⁾ This reverse signaling pathway operates through phosphatidylinositol 3-kinase, mammalian target of rapamycin, and *Runx2*. Furthermore, the apoptosis of osteoclasts leads to the recruitment and

differentiation of osteoblasts. Apoptotic bodies released from osteoclasts also contain RANK and activate RANKL reverse signaling in the same manner as RANK-EVs.⁽⁵⁹⁾

Isolation of extracellular vesicles

The goals of EV isolation are to achieve the highest purity, yield, and a narrow particle-size distribution. Several isolation techniques, based on different principles, have been established for EV isolation. A heterogeneous population of vesicles is often found in most EV isolation methods. The differences in EV quality obtained from each isolation method should be carefully considered.⁽⁹⁷⁾

Ultracentrifugation, or serial centrifugation, is the classic method for isolating EVs based on density, size, and shape. In this method, particles in the suspension are sequentially sedimented depending on the centrifugal force applied, allowing particles to be separated from one another. Ultracentrifugation is one of the most commonly selected methods for EV isolation. However, this approach is time-consuming due to the multiple centrifugation steps required and the need for specialized equipment capable of achieving 100,000g centrifugal forces.

There are two main types of ultracentrifugation: differential ultracentrifugation and density gradient ultracentrifugation. Differential ultracentrifugation typically involves a series of centrifugation cycles with varying centrifugal forces and durations. Particles with high buoyant densities, such as cells, cell debris, and apoptotic bodies, are sedimented first at a low speed (10,000g). The sample is then subjected to repeated ultracentrifugation at above 100,000g to remove non-EV proteins. The resulting suspension is further purified through microfiltration, using filters with pore diameters

of 0.1, 0.22, or 0.45 μm . While this multi-step washing and filtration process yields highly purified EVs, it may also decrease the overall yield.

Density gradient ultracentrifugation uses two approaches to form the gradient: continuous density gradients and stepwise gradients. However, since different EV types can have similar densities, both continuous and stepwise methods may not always result in high purity. The main disadvantage of this method is the potential presence of protein aggregates, apoptotic bodies, and non-EV particles. Nevertheless, density gradient ultracentrifugation typically provides better results in terms of the purity of the EV fraction, as well as the amount of EV proteins and RNA, compared to classical ultracentrifugation.⁽⁹⁸⁾

Sequential filtration is another size-based method consisting of three steps. First, normal filtration is performed using a 100-nm membrane filter to eliminate floating components and cell debris. Next, the filtrate is subjected to tangential flow filtration with 500 kDa molecular weight cut-off hollow fibers. The concentrated retentate is then processed through diafiltration to further deplete contaminants. The third filtration step involves using a 100-nm track-etch filter to enhance EV recovery, with the filters being washed at the end of each step. Sequential filtration allows for the isolation of EVs with high purity and functional integrity, as a result of the low manipulation forces involved.⁽⁹⁸⁾

Currently, several exosome precipitation kits are available for isolating EVs from suspension by altering their solubility or dispersibility. Water-excluding polymers, such as polyethylene glycol, are commonly used in this process. These polymers bind to water molecules, forcing less soluble components out of the solution. Generally, samples are incubated with a precipitation solution at 4°C overnight. The

precipitate containing EVs is then isolated by low-speed centrifugation or filtration. EV precipitation is easy to use and does not require specialized equipment. However, while this technique provides a good yield, it results in a wide range of particle sizes and lower purity.⁽⁹⁹⁾

Extracellular vesicle biogenesis inhibitor

EVs are implicated in various pathophysiological conditions, and in some cases, they contribute to the onset and progression of diseases. As a result, there is growing interest in exploring pharmacological agents that could potentially inhibit the release of EVs. These agents are being investigated both as research tools and as potential therapeutic strategies, with the goal of selectively targeting EVs involved in disease processes while preserving those that perform essential physiological functions.⁽¹⁰⁰⁾ However, the complexity and variability of EV biogenesis present significant challenges in developing a single drug capable of completely blocking EV production. Nevertheless, targeting and inhibiting the primary EV subpopulations linked to specific diseases, if such populations can be identified and effectively targeted, could offer clinical benefits.⁽¹⁰¹⁾

Compounds that influence extracellular vesicle (EV) production can generally be classified into two main categories based on their modes of action: those that primarily affect the trafficking of EVs (such as calpeptin, manumycin A, and Y27632), and those that target lipid metabolism (including pantethine, imipramine, and GW4869).

GW4869 is a small, cell-permeable compound that functions as a selective, non-competitive inhibitor of neutral sphingomyelinase (nSMase), an enzyme involved in

the breakdown of sphingomyelin into ceramide. Studies of the lipid composition of exosomes show an abundant presence of cholesterol, sphingolipids, and ceramide, while phosphatidylcholine is found at lower levels compared to the cellular membrane. This composition is reminiscent of lipid rafts. Additionally, introducing exogenous sphingomyelinase (SMase) or ceramide (C6-ceramide) has been shown to promote the formation of vesicles, underscoring ceramide's crucial role in generating ESCRT-independent extracellular vesicles.⁽¹⁰²⁾ In contrast, GW4869 has been shown to significantly reduce exosome release. The cone-shaped structure of ceramide, produced through SMase activity, is essential for inducing spontaneous curvature, which in turn forms large lipid-raft domains involved in exosome shedding. SMase is present in various cellular compartments, including the Golgi apparatus, endosomes, and cell membrane, making its activity crucial not only for EV formation but also for the shedding of microvesicles.⁽¹⁰³⁾

Nanoparticle size analysis

Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) are commonly used techniques for visualizing and characterizing extracellular vesicles (EVs). These methods rely on the principles of Brownian motion, which involves analyzing the random fluctuations in the light intensity scattered by particles suspended in a solution. Both DLS and NTA are effective for assessing the size distribution of nanoparticles and proteins within the nanometer to low micron range. Each technique has its own advantages. DLS is a quick and simple method that works well for analyzing monodisperse particles and can cover a wide range of particle sizes. In contrast, NTA

offers a more detailed size distribution, with less influence from a small number of larger particles, and also enables the measurement of particle concentration.⁽¹⁰⁴⁾

NTA tracks the Brownian motion of nanoparticles in a solution by directing a laser beam at the sample. A high-sensitivity camera captures the scattered light from the nanoparticles in real time. By analyzing the particle trajectories frame by frame over a defined period, the diffusion coefficient for each particle is calculated based on its movement. Using the Stokes-Einstein equation, the hydrodynamic diameter of the particles is then determined. NTA typically provides accurate measurements of particle size and concentration in solution. The sizes of EVs measured by NTA often align with those obtained by other methods, such as transmission electron microscopy (TEM). However, various factors such as sample dilution, EV purity, and temperature can significantly impact the results. Furthermore, instrument settings, video acquisition, and data analysis parameters must be carefully calibrated to ensure reliable outcomes. NTA is non-invasive and allows for quick data collection, making it an essential tool for evaluating different EV isolation methods, storage conditions, and release processes. Many EV studies rely on NTA as the primary technique for determining EV size and concentration. The scattering mode of NTA generates a high-resolution size distribution profile, which is valuable for characterizing the heterogeneity of EV populations, including exosomes, microvesicles, and apoptotic bodies. However, NTA may face challenges when analyzing exosomes smaller than 50 nm. For consistently sized EVs under 50 nm, dynamic light scattering (DLS) might provide more accurate size and distribution measurements.⁽¹⁰⁵⁾

Monocyte isolation

The monocyte pool in blood is heterogeneous, with a wide range of capacities. Subpopulations of monocytes have been distinguished based on cell surface markers and cellular activities. Adult female donors are preferred for monocyte harvesting due to the protective effect of estrogen against immune cell apoptosis.⁽¹⁰⁶⁾ Based on the relative expression of CD14 and CD16, human monocytes are classified into three subsets: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and nonclassical (CD14⁺CD16⁺⁺) monocytes.⁽¹⁰⁷⁾ Several techniques have been used to isolate monocytes from peripheral blood mononuclear cells (PBMCs). The ideal method should yield a pure monocyte population with minimal contamination from lymphocytes, granulocytes, and platelets. It should also avoid excessive activation of the isolated cells and have minimal impact on their phenotype and function. Previous studies have shown that no single isolation technique is optimal for all scientific purposes, and it is impossible to obtain completely unaltered native monocytes with any of the existing methods.⁽¹⁰⁸⁾

The common methods for monocyte isolation are plastic adhesion and magnetic bead-based immuno-isolation kits (negative and CD14⁺ selection). Plastic adhesion isolation is a simple technique and inexpensive. The monocytes obtained exhibit a similar distribution of subpopulations as PBMCs. Nevertheless, the monocyte yield from plastic adhesion is low compared to immune-based methods, and there is also high contamination with lymphocytes from the plastic adhesion technique. In addition, plastic adhesion may directly activate pro-inflammatory processes.⁽¹⁰⁸⁾

Negative and CD14⁺ selection techniques typically yield high numbers of monocytes without contamination from lymphocytes or granulocytes. However,

negative monocyte selection results in excessive platelet contamination. The distribution of monocyte subpopulations differs significantly from that of PBMCs. On the other hand, CD14⁺ selection is complicated and expensive, and it may lead to notable phenotypic alterations and cell induction.^(108, 109)

For the adhesion method, the type of serum can significantly influence the material surface's effect on monocyte adhesion. Differences in protein components among different serums lead to varying resistance to protein adhesion. Human serum is ideal for monocyte cell adhesion, while fetal bovine serum is suitable for sufficient yield.⁽¹¹⁰⁾

CHAPTER 3

RESEARCH METHODOLOGY

Ethical Approval

The study was conducted under approval of the Institutional Ethics Committee Board, Faculty of Dentistry, Prince of Songkla University (EC6608-043).

Culture of human periodontal ligament cells

The tooth donors were the healthy patient age between 18 - 45 who need surgical removal of third molar. The teeth must have no carious lesion or any developmental abnormality. The exclusion criteria were open apex tooth, fracture of root or separated tooth during surgical procedure. Human PDL cells were isolated from the root surface of third molar teeth (n = 4). After washing several times, the middle third of the root was scraped for harvesting PDL tissue. The scraped tissue was place in 35 mm. disk with culture medium containing Dulbecco Modified Eagle Medium (DMEM; Gibco, Gaithersburg, MD, USA), 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin, and 1% amphotericin B (Gibco) in a humidified chamber with 5% CO₂ at 37°C.

The growth of cells was checked every 48-72 hours using an inverted phase contrast microscope, and medium will be changed every 3 days. After reaching confluence, the adherent confluent cells were detached and transferred to centrifugated at 2,000g for 5 minutes. The medium was discarded and the pellets of cells was sub-

cultured in T75 culture flasks. Cells from third and fifth passages were used in the subsequence experiments.⁽¹¹¹⁾

To characterize the PDL cells, cells were collected and stained with mesenchymal cell surface markers, including APC-conjugated anti-human CD73, Alexa Fluor® 700-conjugated anti-human CD90, and APC/Fire™ 750-conjugated anti-human CD105 (BioLegend, San Diego, CA, USA). FITC-conjugated anti-human CD34 and PC5-conjugated anti-human CD45 (Beckman Sciences, Marseille, France), Coulter Life markers for hematopoietic stem cells, were stained for a negative control. Expressions of surface markers were analyzed using a flow cytometer (CytoFLEX SRT, BECKMAN COULTER, Brea, CA, USA) together with the CytExpert SRT software version 1.1 (BECKMAN COULTER). As a positive control for osteogenic inductions of human PDL cells, they were treated with osteogenic induction medium, containing complete medium supplemented with 50 mg/ml ascorbic acid (Sigma-Aldrich, Darmstadt, Germany), 10 mM β -glycerophosphate (Sigma-Aldrich), and 100 nM dexamethasone (Sigma-Aldrich).

Monocyte isolation & culture for human osteoclasts

To isolate PBMCs for osteoclastogenesis, a 20-ml volume of venous blood was collected from healthy volunteers (n = 10) and diluted with phosphate-buffered saline (PBS) at a ratio of 1:1. The diluted blood was layered on top of Ficoll (Cytiva Sweden AB, Sweden) density gradient medium and centrifugated at 400g for 30 minutes at room temperature without deceleration. PBMCs were collected from the buffy coat and resuspended in complete α MEM medium (Gibco), 10% FBS (Gibco), and 1% penicillin-streptomycin at 2×10^7 cells/ml. On the following day, non-adherent cells

were removed by thorough washing with PBS. The adherent cells were approximately 6×10^4 cells/cm².

To induce osteoclastogenesis, PBMCs were cultured in the complete α MEM medium, supplemented with 15 ng/ml of recombinant human macrophage colony stimulating factor (M-CSF; Stemcell Technologies, Vancouver, Canada) and 30 ng/ml of recombinant human RANKL (Stemcell Technologies) with medium replacement every 3 days.

Characterization of mature osteoclasts

To characterize the mature osteoclasts, PBMCs were seeded on cover slips in 48-well plates. Osteoclastogenesis was induced with 15 ng/ml of M-CSF and 30 ng/ml of RANKL in complete α MEM medium for 9 days. After maturation of osteoclasts, the cells were fixed with 4% formaldehyde (Sigma-Aldrich) in PBS for 15 minutes at 4°C, then washed twice with PBS. For TRAcP staining, cells were incubated with 1.5 mM of Fast Red Violet LB salt (Sigma-Aldrich) for 2 hours at 37°C. Washed cells 2 times with PBS for 5 minutes each. The images were captured using an inverted phase contrast microscope.

The actin ring formation of mature osteoclasts was determined by specific fluorescent staining. PBMCs were seeded on glass slides in 48-well plates with 15 ng/ml of M-CSF and 30 ng/ml of RANKL for 9 days. The cells were fixed with 4% paraformaldehyde in PBS and reacted with 20 nM of Alexa Fluor[®] 488-conjugated phalloidin in 0.05% Triton X-100 and 3% bovine serum albumin (BSA) in PBS at room temperature for 1 hour. Subsequently, cells were stained with 1 μ M of 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes. Then, the glass slides were mounted with

DAKO fluorescence mounting medium, and the stained cells were visualized by a fluorescence microscope and their images were captured under a fluorescence microscope (ECLIPSE Ni-E, Nikon, Tokyo, Japan).

Preparation of conditioned medium from mature osteoclasts

The conditioned medium from mature osteoclasts (OC-CM) at days 9, 12, 15, and 18 was collected, pooled, centrifuged at 2,000g for 10 minutes to remove cell debris, and then concentrated by 20-fold using centrifugation at 4,000g with the Amicon Ultra Filter Unit (ref: UFC910024, 100kDa Merk Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) for 15 minutes at 4°C.⁽⁵⁷⁾ The 20-fold concentrated OC-CM was kept at -80°C for further use.

Preparation of conditioned medium from mature osteoclasts treated with extracellular vesicle release inhibitor

GW4869 was first dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific) to obtain 5 mM stock solution. The stock was then diluted in the culture medium to reach final concentration of 20 μ M GW4869 (final concentration of DMSO in the culture medium was 0.005%).⁽¹¹²⁾

The cytotoxic effects of DMSO and GW4869 on mature osteoclasts were evaluated by a fluorometric assay to measure the cell metabolic activity using alamarBlue dye (Bio-Rad Laboratories, Hercules, CA, USA). PBMCs were seeded in 96-well sterile black plates with clear bottom and treated with 15 ng/ml of M-CSF and 30 ng/ml of RANKL for 9 days until maturation of osteoclasts. Mature osteoclasts on day 9 were treated with 0.05% DMSO or 20 μ M of GW4869 for 9 days. Then, a 10- μ L

volume of alamarBlue dye was added to each well and incubated at 37°C for 3 hour. The fluorescence signal was measured by a multimode microplate reader (EnVision® 2105, Perkin Elmer, Shelton, CT, USA) with excitation and emission wavelengths at 530 and 590 nm, respectively. The signal from DMSO and GW4869 untreated cells was set at 100% of cell viability.

To inhibit EVs' release, mature osteoclasts on day 9 were treated with 20 μ M of GW4869 for three days, and the conditioned medium from GW4869-treated mature osteoclasts (GW-OC-CM) were collected from days 12, 15, and 18, and then concentrated by 20-fold following the protocol as described above.

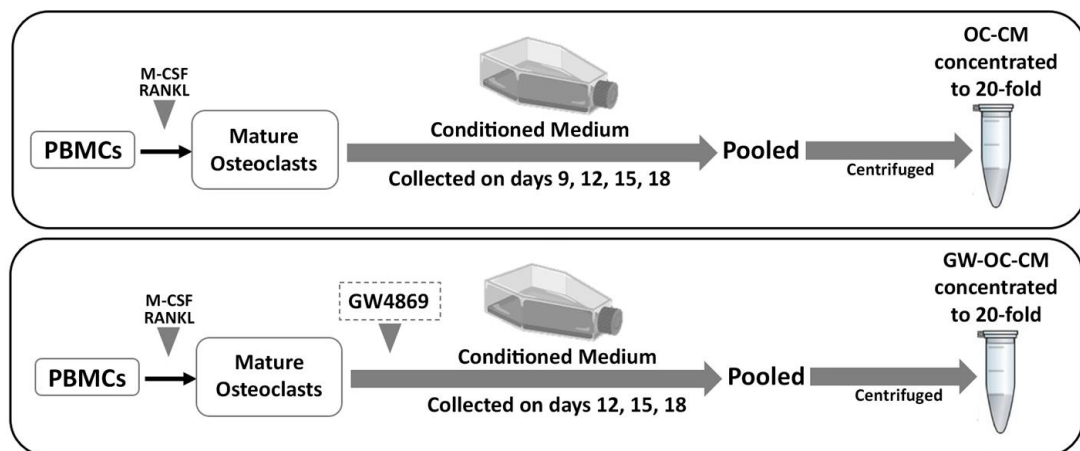


Figure 1 Schematic diagram showing the preparation of conditioned medium from mature human osteoclasts and GW4869-treated mature human osteoclasts

Isolation of extracellular vesicles from osteoclast conditioned medium

Osteoclasts at day 6 were washed three times with PBS and culture medium were replaced with α MEM containing 10% EV-depleted FBS (Gibco) and 1% penicillin-streptomycin. OC-CM was collected and pooled from days 9, 12, 15, and 18. The pooled OC-CM was kept at -80°C prior to EV isolation.

Osteoclasts' extracellular vesicles (OC-EVs) were isolated through a series of microfiltration and ultracentrifugation steps. First, OC-CM was initial centrifuged at 300g for 5 minutes at 4°C to remove cells, followed by centrifugation at 2,000g for 30 minutes at 4°C to remove cellular debris then passed through a 0.22- μ m vacuum filter (Corning® bottle-top vacuum filter system, Merck). The filtrate was ultracentrifuged at 100,000g for 90 minutes at 4°C by ultracentrifuge machine with SW 41 Ti rotor (Optima XPN-100, Beckman Coulter, Inc. CA, US). The pellets of EVs were resuspended with phosphate-buffered saline (PBS; equivalent to 20-fold concentration of initial OC-CM; 60 ml OC-CM became 3 ml PBS per 1 cycle) and stored at -80°C for further use.

For preparation of OC-EVs from GW4869 treated osteoclasts (GW-OC-EVs), GW-OC-CM was processed through a series of microfiltration and ultracentrifugation as mentioned above.

Characterization, quantification, and size distribution of extracellular vesicles

For TEM analysis of OC-EVs, 10 μ l of EV suspension in PBS was deposited onto 400 mesh carbon-coated copper grid and left to air-dry for 3 minutes. Grid was then washed with PBS and fixed by transfer onto 1% glutaraldehyde for 5 minutes. After washing with distilled water, samples were stained with 10 μ l of 1% uranyl

acetate solution (ProSciTech, Queensland, Australia) for 2 minutes. Finally, grid was air-dry for 10 minutes and observed under TEM (Talos F200X G2, Thermo Fisher Scientific, MA, US) for visualization of morphological characteristics and diameter measurements.

Absolute size distribution and number of OC-EVs were direct tracked by NTA. Isolated OC-EVs were diluted to 10-fold and analyzed in the nanoparticle tracking analyzer (NanoSight NS300, Malvern Panalytical, Malvern, UK). Isolated GW-OC-EVs were also analyzed with NTA.

Western blot analysis for protein marker of extracellular vesicles

Western blot analysis was used for detection of EVs' transmembrane proteins (CD9, CD63 and CD81) and EV cytosolic protein (TSG101). Calnexin (endoplasmic reticulum protein) and Lamin A/C (nuclear protein) were negative control. GAPDH was used as an internal control. For comparison with EVs' proteins, total proteins of mature osteoclasts were extracted with RIPA buffer. Protein concentrations were measured using multi-mode microplate reader. Equivalent amounts of proteins were separated on SDS-PAGE and transfer onto PVDF membranes. The blotting membrane were blocked using 5% bovine serum albumin for 2 hours at room temperature and incubated with primary antibodies at 4°C overnight. After three PBS washes, membranes were incubated with secondary antibodies at 37°C for 1 hour. ECL solution was then prepared, and the bands on the membranes were scanned and imaged in a dark room.

Flow cytometry analysis of lipid bilayer membrane and RANK on OC-EVs

For investigation of lipid bilayer membrane, pellets in 1% PBS were stained with 3 μ M of ACO-600 membrane labelling dye (Acoerela Pte, Ltd., Singapore) for 1 hours at 37°C in the dark. For the dye control, replaced OC-EVs with 1% PBS. Removed excess dye from samples (OC-EVs and dye control) by ultra-filtration using a 100 KDa Amicon Ultra Filter Unit (Merk Millipore Ltd) and centrifuged at 3,000g for 10 minutes to re remove the free dye. Repeated wash step 2 times by adding 1%PBS to the Amicon unit. After washing, resuspended stained samples for flow cytometry analysis.

For analysis of RANK on EVs' membrane, OC-EVs in 1% PBS were stained with phycoerythrin (PE)-conjugated anti-human RANK antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 1:500 for 30 minutes at 4°C, Expression of RANK was analyzed using a flow cytometer (CytoFLEX SRT).

RT-qPCR

To evaluate osteogenic gene expressions, PDL cells were seeded at 5×10^5 cells per well in 6-well plates. At 70-80% cell confluence, the cells were treated according to protocols as mentioned below. The level of mRNA expressions of *RUNX2*, *OSX*, *BMP-2*, *COL1A1*, *OCN*, and *BSP* were determined by RT-qPCR. In brief, the amount of total RNA extracted by the E.Z.N.A.® Total RNA kit (Omega BIO-TEK, Norcross, GA, USA) was quantified by NanoDrop One™ (Thermo Fisher Scientific, Carlsbad, CA, USA) at wavelengths of 260 nm. and 280 nm. The ratios of A260 to A280 were found to be greater than 2.0 in all RNA samples.

Complementary deoxyribonucleic acid (cDNA) was synthesized from 200-ng amounts of each RNA sample using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) according to the manufacturer's instructions. PCR was performed using the CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories, Inc., Berkeley, CA, USA) for 45 cycles at 95 °C for 20 seconds, 60 °C for 20 seconds, and 72 °C for 25 seconds. Each 20 µl of PCR mixture contained 5 µl of cDNA, 0.8 µl of each forward and reverse primer at 10 µM, 10 µl of SYBR green mix (SENSIFAST SYBR NO-ROX KIT; Thermo Fisher Scientific), and 3.4 µl of nuclease-free water. The sequences of oligonucleotide primers are summarized in Table 2. The melting curve analysis was performed for each PCR run and showed a single peak of PCR product without non-specific amplification of primer dimer. The messenger ribonucleic acid (mRNA) expressions of target genes were quantified by the $2^{-\Delta\Delta C_t}$ method using *GAPDH* mRNA expression as a housekeeping gene control for the target gene expression.

Table 2 Oligonucleotide primers used in this study

Gene	Primer sequences (5'-3')		Accession number
<i>GAPDH</i> ⁽³³⁾	F	TCA TGG GTG TGA ACC ATG AGA A	NM_002046.3
	R	GGC ATG GAC TGT GGT CAT GAG	
<i>RUNX2</i> ⁽³³⁾	F	ATG ATG ACA CTG CCA CCT CTG A	NM_001024630.3
	R	GGC TGG ATA GTG CAT TCG TG	
<i>OSX</i> ⁽³³⁾	F	GCC AGA AGC TGT GAA ACC TC	NM_152860.1
	R	GCT GCA AGC TCT CCA TAA CC	
<i>BSP</i> ⁽³³⁾	F	GCG TGA AAA GAG AGA CTG C	NM_001200.2
	R	CCA TTG AAA GAG CGT CCA C	
<i>OCN</i> ⁽³³⁾	F	CTT TGT GTC CAA GCA GGA GG	NM_199173.4
	R	CTG AAA GCC GAT GTG GTC AG	
<i>COL1A1</i> ⁽³³⁾	F	GTG CTA AAG GTG CCA ATG GT	NM_000088.3
	R	ACC AGG TTC ACC GCT GTT AC	
<i>BSP</i> ⁽¹¹³⁾	F	AAG CAA TCA CCA AAA TGA AGA CT	NM_004967
	R	TGG AAA TCG TTT TAA ATG AGG ATA	

Abbreviations: F – forward, R – reverse

Cell staining for osteogenic differentiation and biomineralization

To perform cell staining for osteogenic differentiation and biomineralization, PDL cells were seeded at 5×10^5 cells per well in 96-well plates. At 50-60% cell confluence, the cells were treated according to protocols as mentioned below.

Alkaline phosphatase staining

The PDL cells were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) in PBS for 15 minutes and washed twice with PBS. An amount of 0.5 ml of CHAP buffer, which included 100 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM MgCl_2 in distilled water, was added to the fixed cells for 30 minutes at room temperature in the dark. After removal of the buffer, the cells were stained with 0.5 ml of 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt/4-nitroblue tetrazolium chloride solution (Ameresco, OH, USA) in distilled water for 30 minutes at room temperature in the dark followed by washing twice with PBS for 5 minutes each. The digital images of ALP staining were captured by a stereomicroscope (Olympus SZ61; Olympus, Inc., Tokyo, Japan), and the stained areas were assessed by the ImageJ program ((National Institutes of Health, Bethesda, MD, USA).

Alizarin red staining

Biomineralization was determined by Alizarin red staining and von Kossa staining to monitor calcium and phosphate ion deposits, respectively. The fixed cells were stained with 2% (v/v) of Alizarin red solution (Ameresco), pH 4.1–4.3, for 45 minutes at room temperature. Thereafter, the cells were rinsed several times with distilled water to remove the remaining stains followed by rinsing with 0.5 ml of PBS to stop the reaction.

A quantitative analysis of Alizarin red staining was achieved by adding 0.2 ml of 10% (v/v) acetic acid to each well and incubating for 30 minutes at room temperature with shaking to solubilize the red stains. A cell scraper was used to scrape all the cells in the well, and the mixture was then transferred to a 1.5-ml centrifuge tube. The mixture was vigorously vortexed for 30 seconds, and the tube was sealed with parafilm, heated at 85 °C for 10 minutes, and incubated on ice for 5 minutes before centrifugation at 12,000g for 15 minutes. Subsequently, 100 µl of the supernatant was transferred to a new 1.5-ml centrifuge tube, and 30 µl of 10% (w/v) ammonium hydroxide was added. A 100-µl aliquot was pipetted and transferred to a 96-well black plate with a clear bottom to measure the absorbance at a wavelength of 405 nm. by a multimode microplate reader (PerkinElmer®, Hamburg, Germany).

von Kossa staining

von Kossa staining was performed by adding 0.5 ml of a 1% (w/v) silver nitrate solution to the fixed cells and incubating for 3 minutes at room temperature in the dark. The solution was then aspirated and the cells were rinsed 3 times with distilled water followed by the addition of 0.5 ml of 5% (w/v) sodium carbonate in 10% (v/v) formaldehyde for 3 minutes at room temperature in the dark. The cells were then rinsed with distilled water 3 times, and 0.5 ml of 5% (w/v) disodium thiosulphate solution was added for 3 minutes at room temperature in the dark followed by washing with distilled water 3 times. The digital images of von Kossa staining were captured by a stereomicroscope (Olympus SZ61; Olympus, Inc., Tokyo, Japan), and the stained areas were assessed by the ImageJ program ((National Institutes of Health, Bethesda, MD, USA).

The osteogenic induction of periodontal ligament cells by osteoclast conditioned medium

The PDL cells were treated with concentrated OC-CM at a ratio of 1:10 (v/v) in osteogenic induction medium, or left untreated as a negative control. Culture medium with or without OC-CM was refreshed every 3 days. The expression of osteogenic-related genes (*RUNX2*, *OSX*, *OCN*, and *COL1A1*) (*GAPDH* as an internal control) was analyzed using RT-PCR on days 1 and 7. On day 7, ALP staining was performed. Biomineralization was detected by Alizarin red and von Kossa staining on day 14.

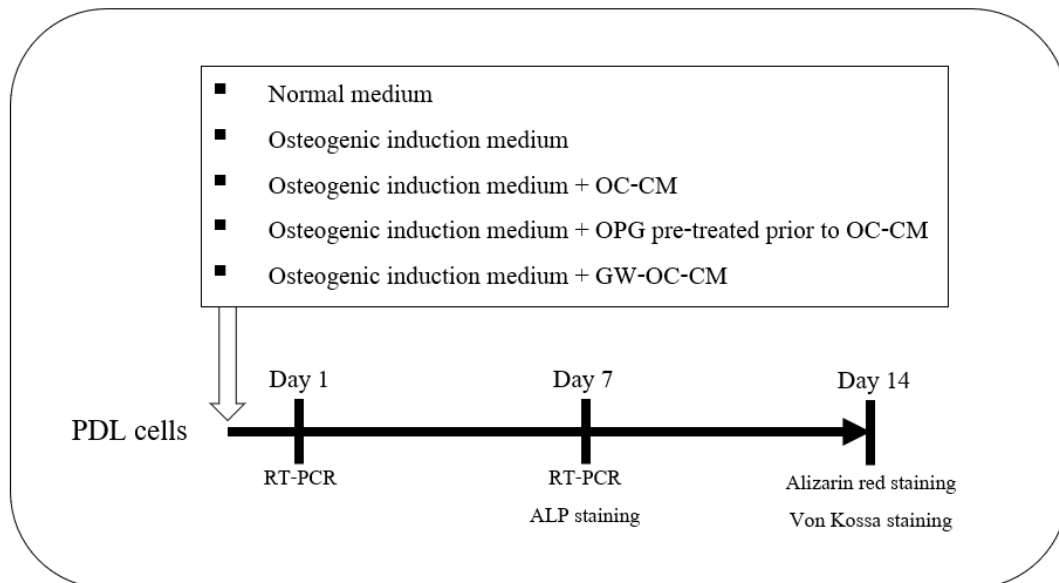
Inhibition of RANK-RANKL signaling for osteogenic induction of osteoclast conditioned medium treated periodontal ligament cells

The PDL cells were pre-treated with 10 ng/ml of recombinant human OPG (Stemcell Technologies) for 1 hour. The cells were rinsed with 1% PBS, and the medium was refreshed before OC-CM treatment. Culture medium with or without OC-CM or OPG was refreshed every 3 days. The expression of osteogenic-related genes (*RUNX2*, *OSX*, *OCN*, and *COL1A1*) (*GAPDH* as an internal control) was analyzed using RT-PCR on days 1 and 7. On day 7, ALP staining was performed. Biomineralization was detected by Alizarin red and von Kossa staining on day 14.

Investigation of the role of extracellular vesicles in osteoclast conditioned medium on osteogenic induction of periodontal ligament cells

To investigate the role of EVs from osteoclast conditioned medium, the PDL cells were treated with concentrated GW-OC-CM at a ratio of 1:10 (v/v) and analyzed for osteogenic gene expression and biomineralization in comparison with those treated

with OC-CM. The expression of osteogenic-related genes (*RUNX2*, *OSX*, *OCN*, and *COL1A1*) (*GAPDH* as an internal control) was analyzed using RT-PCR on days 1 and 7. On day 7, ALP staining was performed. Biomineralization was detected by Alizarin red and von Kossa staining on day 14.



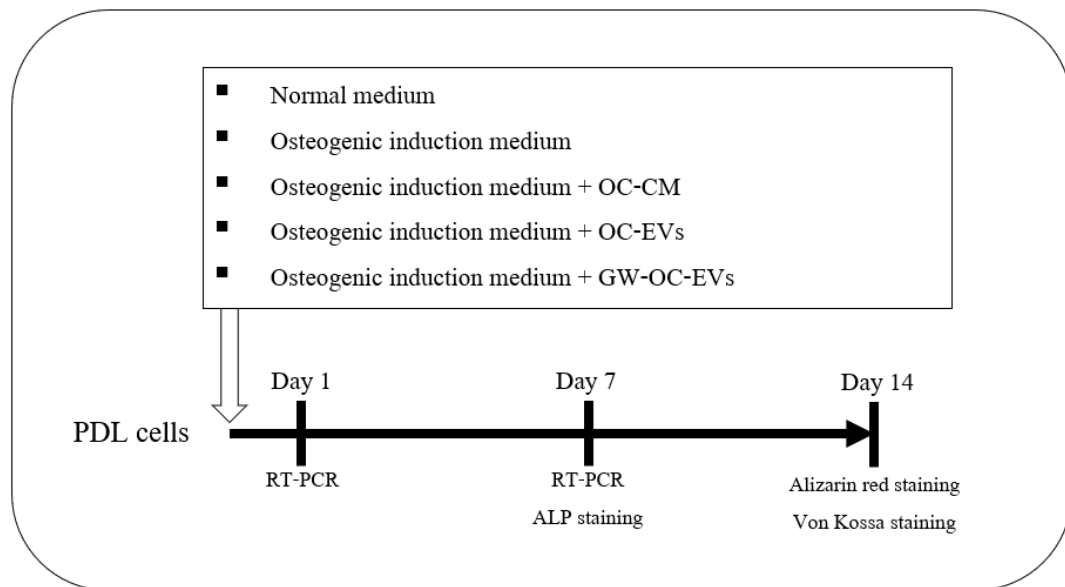
Co-localization of RANK and RANKL

Co-localization of osteoclasts' RANK-EVs and RANKL on PDL membrane was determined. The PDL cells were seeded on a glass slide at 1×10^5 cells/ml and then treated with OC-CM or GW-OC-CM at the ratio 1:10 of osteoclast conditioned medium to PDL cell culture medium of 1:10 (v/v) for 1 hour or left untreated as a negative control. The cells were then fixed with 4% (v/v) paraformaldehyde in PBS for 15 minutes and washed twice with PBS. The cells were permeabilized for 30 minutes at room temperature with 0.1% (v/v) Triton X-100 (Ameresco) in PBS that contained 3% (w/v) bovine serum albumin. The cells were reacted with phycoerythrin (PE)-conjugated anti-human RANK antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 1:200 and Alexa Fluor® 488-conjugated anti-human RANKL antibody (Santa Cruz Biotechnology, Inc.) at 1:100, and then stained with DAPI at 1:200 for 2 hours in the dark at room temperature. The cells were then washed three times with PBS, mounted with the fluorescence mounting medium (DAKO Corporation, Carpinteria, CA, USA), and observed under the fluorescence microscope.

Elucidation of the role of osteoclasts' extracellular vesicles on osteogenic differentiation of periodontal ligament cells

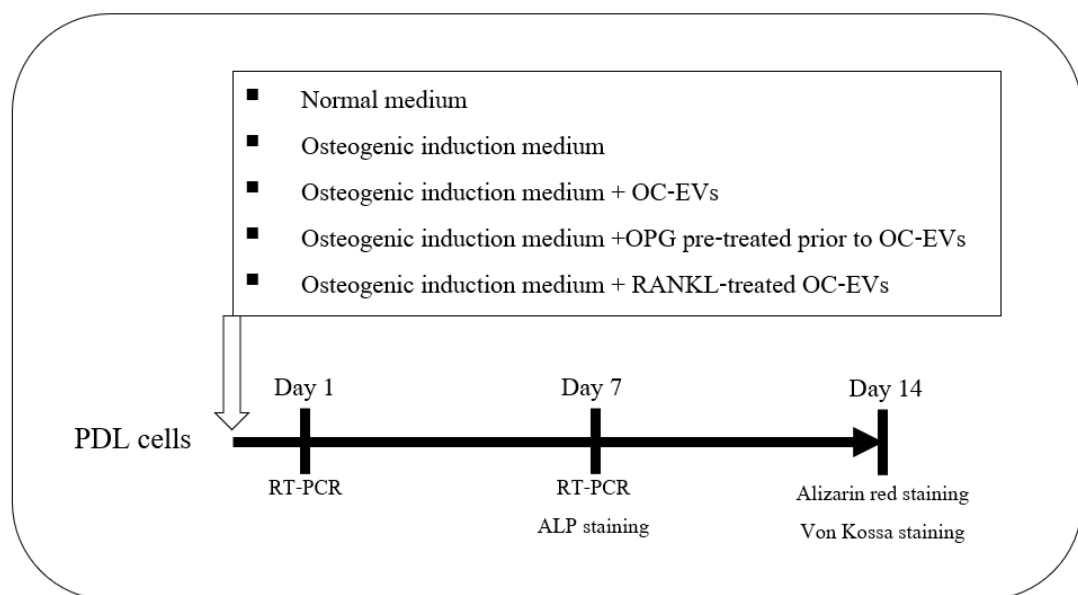
To investigate the role of isolated EVs from osteoclast conditioned medium, the PDL cells were treated with the isolated OC-EVs (approximately $1,250 \times 10^6$ particles in 1 ml of culture medium) or GW-OC-EVs (equivalent to OC-CM at the ratio of 1 to 10 (v/v) in culture medium). As a positive control, the PDL cells were treated with OC-CM at a ratio of 1:10 (v/v). The culture medium was refreshed every 3 days. The expression of osteogenic-related genes (*RUNX2*, *OSX*, *OCN* and *COL1A1*) (*GAPDH* as

internal control) were analyzed using RT-PCR on days 1 and 7. On day 7, ALP staining was performed. Biom mineralization was detected by Alizarin red and von Kossa staining on day 14.



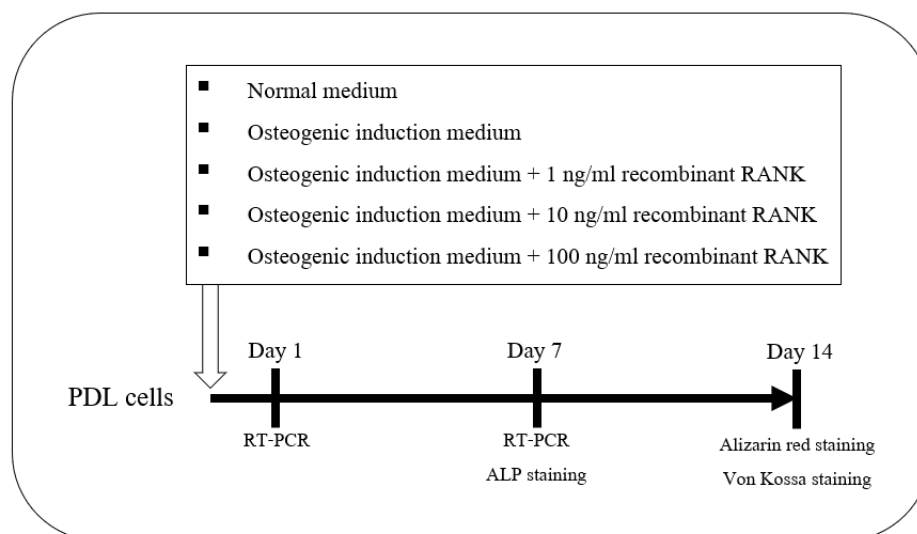
Investigation of RANK - RANKL signaling for osteogenic induction of periodontal ligament cells treated with osteoclasts' extracellular vesicles

To inhibit membranous RANKL on the PDL cells, the cells were pre-treated with 10 ng/ml of recombinant human OPG for 1 hour before OC-EVs treatment. To inhibit RANK on EVs, OC-EVs were treated with 10 ng/ml of recombinant human RANKL for 1 hour before PDL cell treatment. As a positive control, the PDL cells were treated with OC-EVs only. The concentration of OC-EVs was the same as described above. The culture medium was refreshed every 3 days. The expression of osteogenic-related genes was analyzed using RT-PCR on days 1 and 7. On day 7, ALP staining was performed. Biomineralization was detected by Alizarin red and von Kossa staining on day 14.



Effect of recombinant RANK on the osteogenic differentiation and biomineralization of periodontal ligament cells

The PDL cells were treated with 1, 10, or 100 ng/ml recombinant human RANK in osteogenic induction medium (complete medium supplemented with 50 mg/ml ascorbic acid, 10 mM β -glycerophosphate, and 100 nM dexamethasone), or left untreated in normal medium as a negative control. The medium and treatment were refreshed every other day. The expression of osteogenic-related genes was analyzed with RT-PCR on days 1 and 7. On day 7, ALP staining was performed. Biomineralization was detected by Alizarin red and von Kossa staining on day 14.



Statistical analysis

All experiments were performed in triplicate for each of the four PDL cell lines. The Shapiro-Wilk test was used to check the normality of the data distribution. One-way analysis of variance followed by Student–Newman–Keuls post hoc test was used to determine the significant difference between the groups at $p < 0.05$. The statistical analysis was performed using SPSS software version 26.0 for Windows.

CHAPTER 4

RESULT

Characterization of human periodontal ligament cells

The harvested periodontal ligament cells showed a spindle-shaped morphology (Fig 2A), were negative for expression of the hematopoietic cell marker, CD34 or CD45 (Fig 2B or C), but were positive for expressions of mesenchymal cell markers, CD73, CD90, and CD105 (Fig 2D-F).

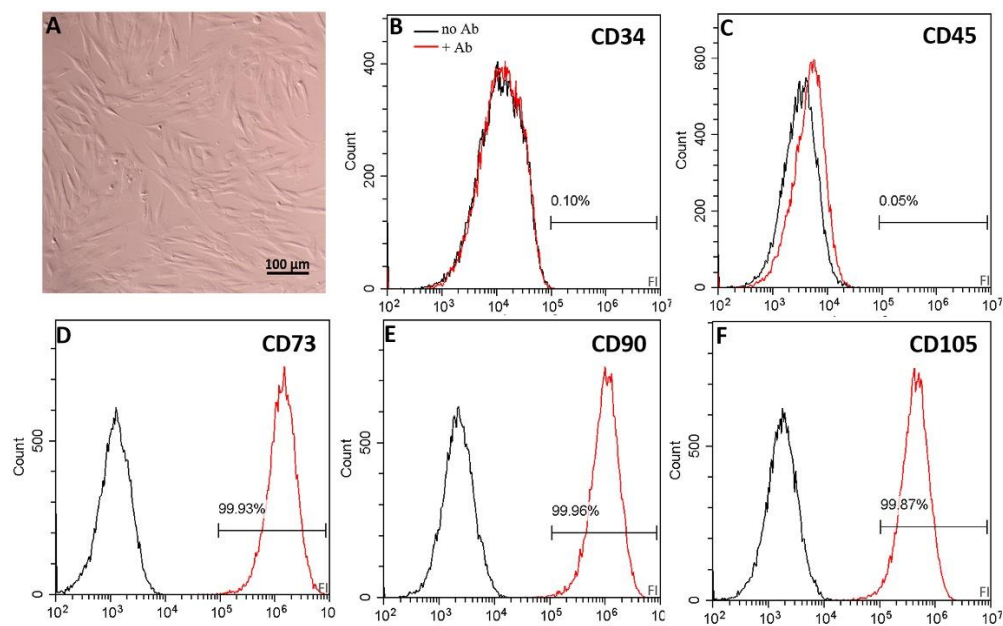


Figure 2 Characterization of human periodontal ligament cells

Characterization of human mature osteoclasts

The mature osteoclasts were positive for TRAcP staining (Fig 3A). The immunofluorescence elucidated containing of multiple nuclei (Fig 3B), and actin ring formation (Fig 3C).

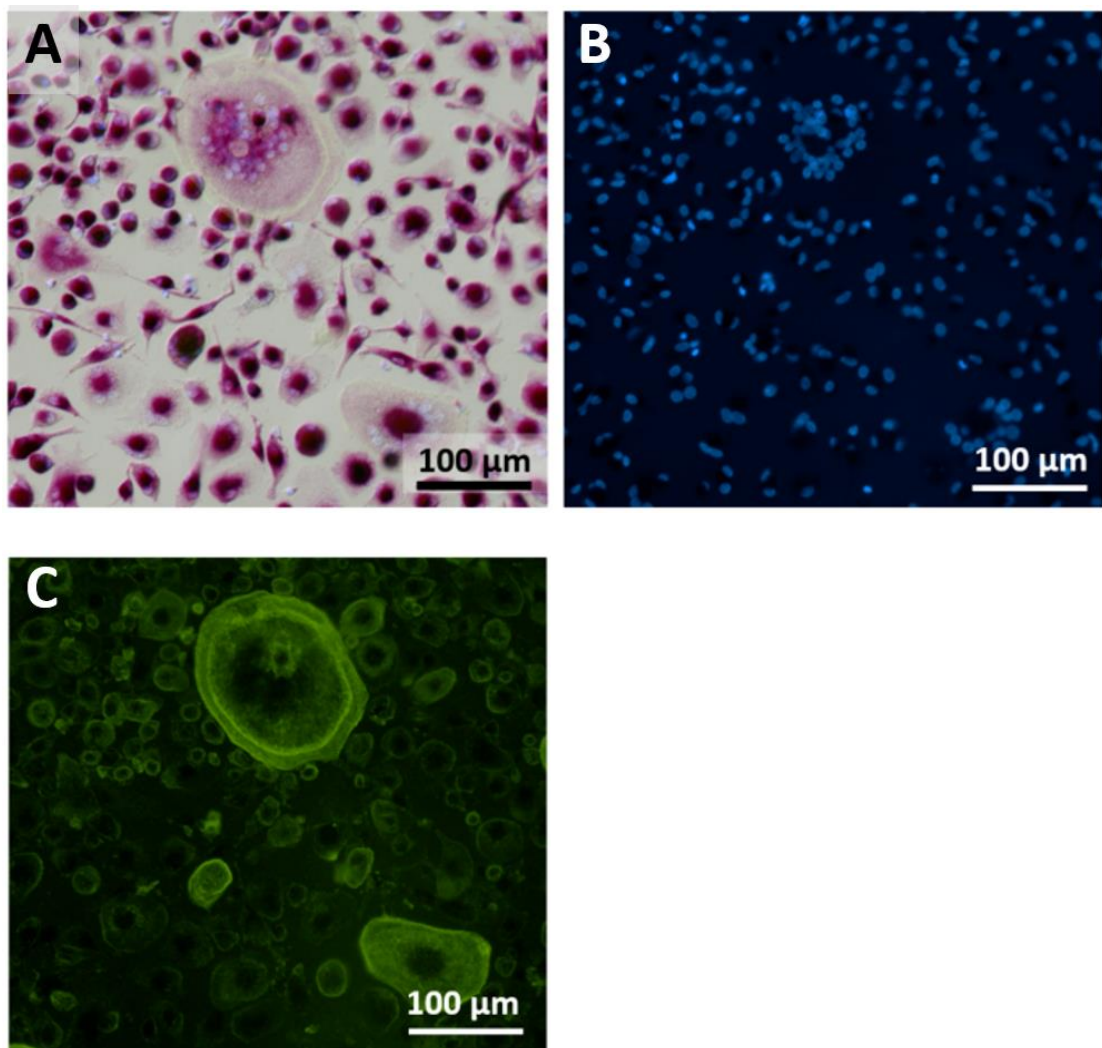


Figure 3 Characterization of mature human osteoclasts showing positive staining of tartrate-resistant acid phosphatase (red in A), multinucleated cells (blue in B), and actin ring formation (green in C).

Identification of osteoclasts' extracellular vesicles

The particles isolated from OC-CM or GW-OC-CM by ultracentrifugation were identified for the presence of EVs, morphology, size distribution, and RANK expression.

Detection for lipid bilayer membranous particles

The particles isolated from OC-CM by ultracentrifugation were initially identified by flow cytometry using ACO-600 dye to detect the lipid bilayer membrane. Flow cytometry analysis demonstrated that the proportion of lipid bilayer membranous particles was 59.31% of the total (Fig 4).

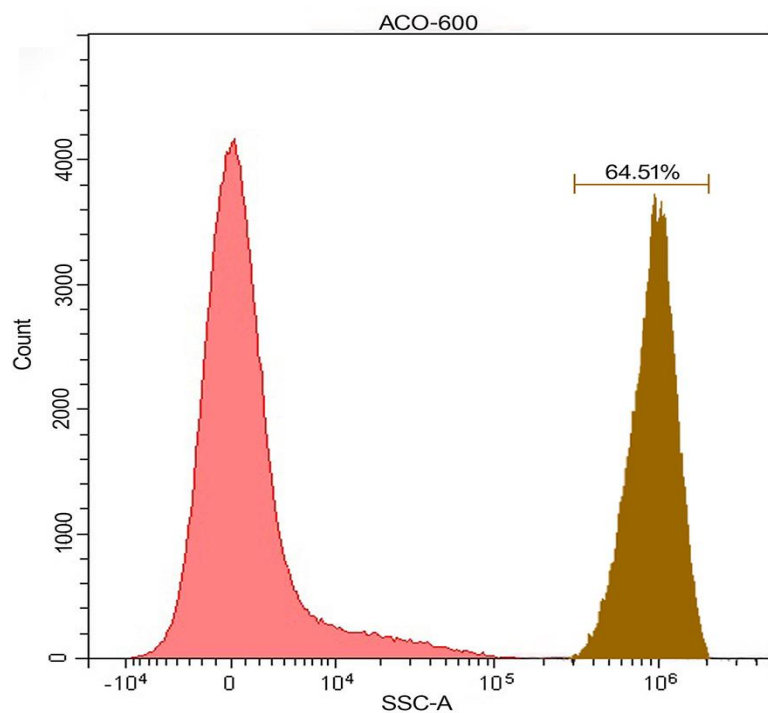


Figure 4 Flow cytometry diagram showed total particles (100%; pink) isolated from osteoclast conditioned medium by ultracentrifugation, the proportion of lipid membranous particles was 64.51% (brown).

Transmission electron microscopy of extracellular vesicles

The images of particles isolated from OC-CM by ultracentrifugation were captured by TEM. The particles showed round and ovoid in shape. The diameters were approximately 80-100 nm. The particles exhibited a bilayer membrane structure (Fig 5).

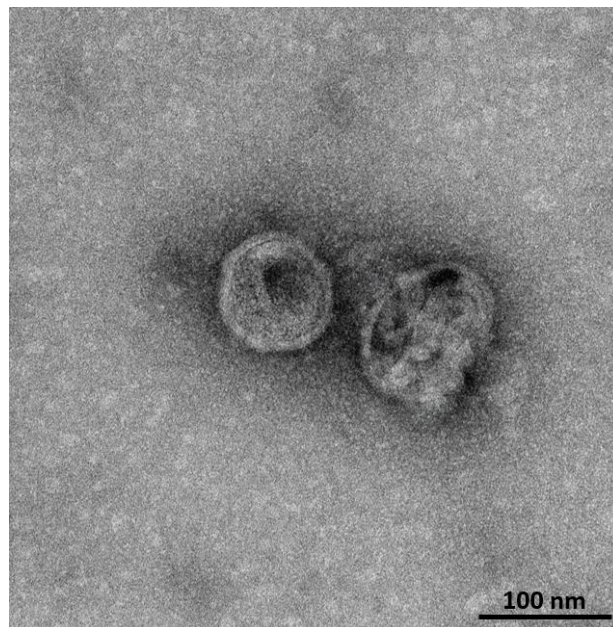


Figure 5 The morphology of osteoclasts' extracellular vesicles was elucidated by transmission electron microscopy. The image showed the bilayer membrane structure of the extracellular vesicles.

Protein markers of extracellular vesicles compared with osteoclast cell lysate

GAPDH (molecular weight of 37 kDa) and CD9 (24 kDa) were run on a 12% gel. GAPDH protein from both osteoclast cell lysate and OC-EVs was detected, but less GAPDH was found in OC-EVs. Similarly, CD9 protein from osteoclast cell lysate was detected more than CD9 protein from OC-EVs (Figure 6).

TSG101 (molecular weight of 45 kDa) and Lamin A/C (62 and 69 kDa) were run on a 10% gel. TSG101 protein from OC-EVs appeared slightly, while TSG101 protein from osteoclast cell lysate was more prominent. Lamin A/C protein from osteoclast cell lysate was detected at two molecular weights, while Lamin A/C protein from OC-EVs was not found (Figure 6).

CD63 (molecular weight of 30–60 kDa) and CD81 (22 kDa) were run on a 12% gel. CD63 and CD81 proteins from osteoclast cell lysate were clearly shown, while CD63 and CD81 proteins from OC-EVs were less detected (Figure 6).

Calnexin (molecular weight of 90 kDa) was run on a 7% gel. Calnexin protein from osteoclast cell lysate was clearly visible, while Calnexin protein from OC-EVs was absent (Figure 6).

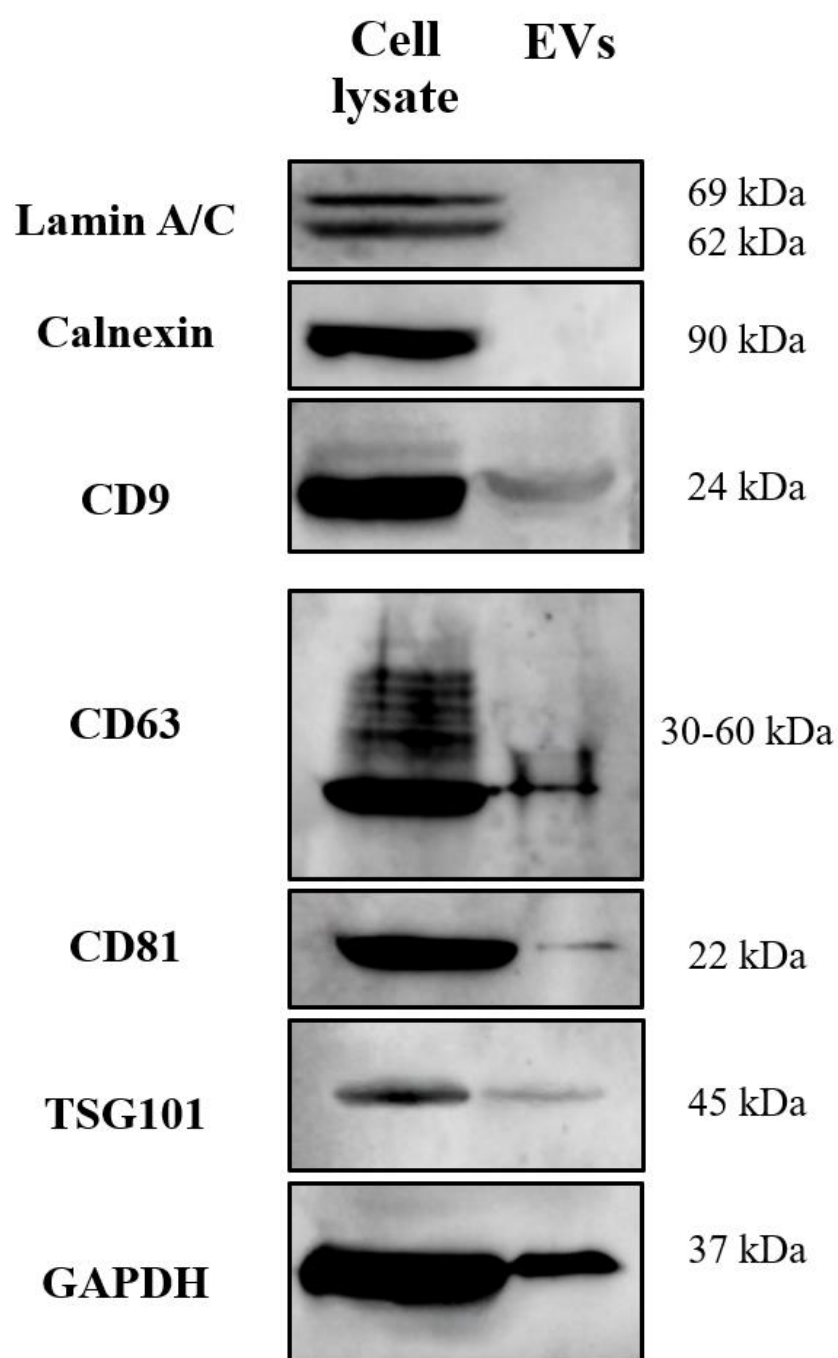


Figure 6 The images of Western blot analysis of extracellular vesicle protein markers from osteoclast cell lysate and osteoclast extracellular vesicles.

Quantification and size distribution of extracellular vesicles

OC-EVs and GW-OC-EVs were performed NTA. The concentration of OC-EVs resuspended in 1% PBS was $12,500 \times 10^6$ particles/ ml. The average size of particles was 100.7 nm. The most common particle size was 77.3 nm in diameter (Figure 7). The concentration of GW-OC-EVs resuspended in 1% PBS was 93.8×10^6 particles/ ml. The average size of the particles was 160.5 nm with a wide range of standard deviation (Figure 8).

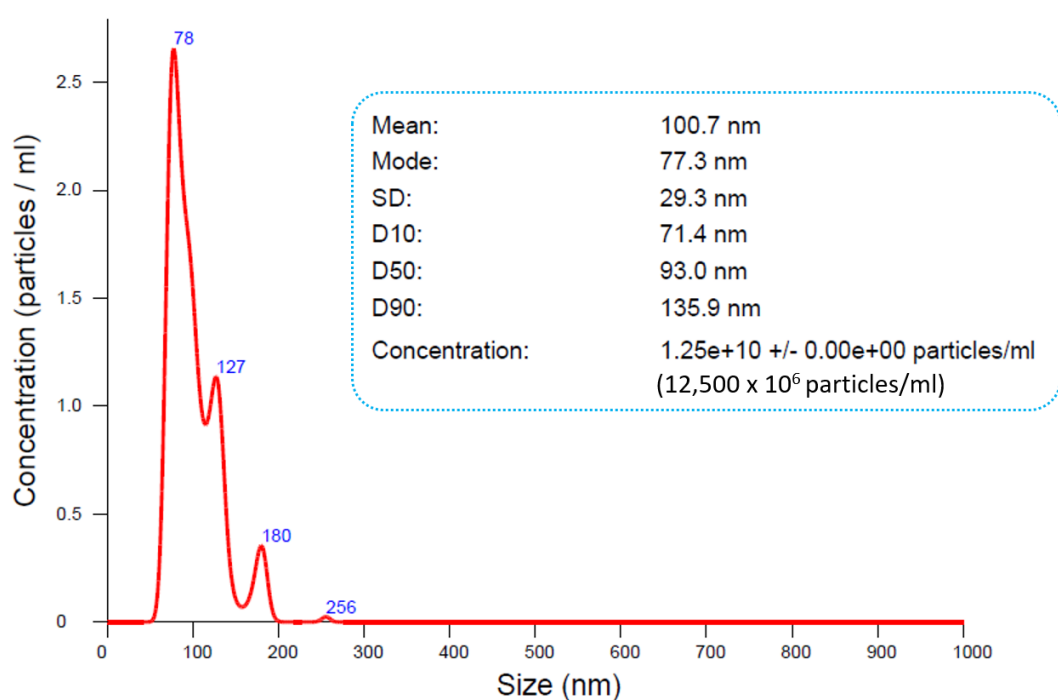


Figure 7 The diagram showed the quantity and size distribution of OC-EVs particles.

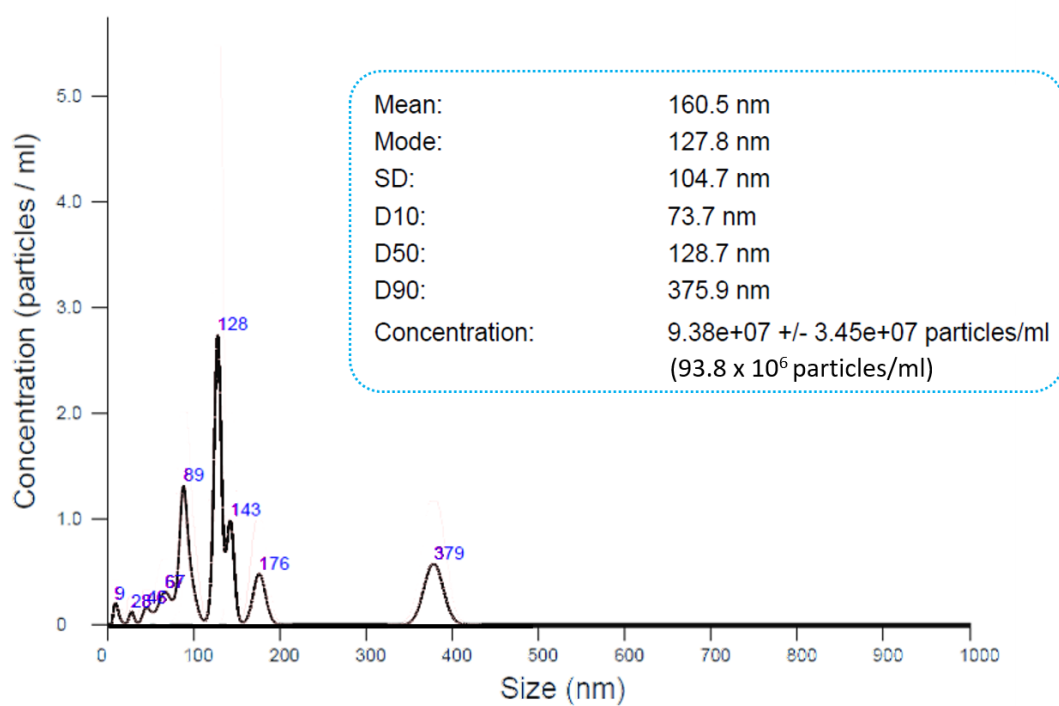


Figure 8 The diagram showed the quantity and size distribution of GW-OC-EVs particles.

Identification of RANK on osteoclasts' extracellular vesicles

The percentage of vesicles positive for RANK was found at 7.94 (Figure 9A). To validate the positive result of flow cytometry, the pellet was incubated with either PBS (Figure 9B) or with the isotype control antibody, PB1 (Figure 9C), showed no immunoreactivity.

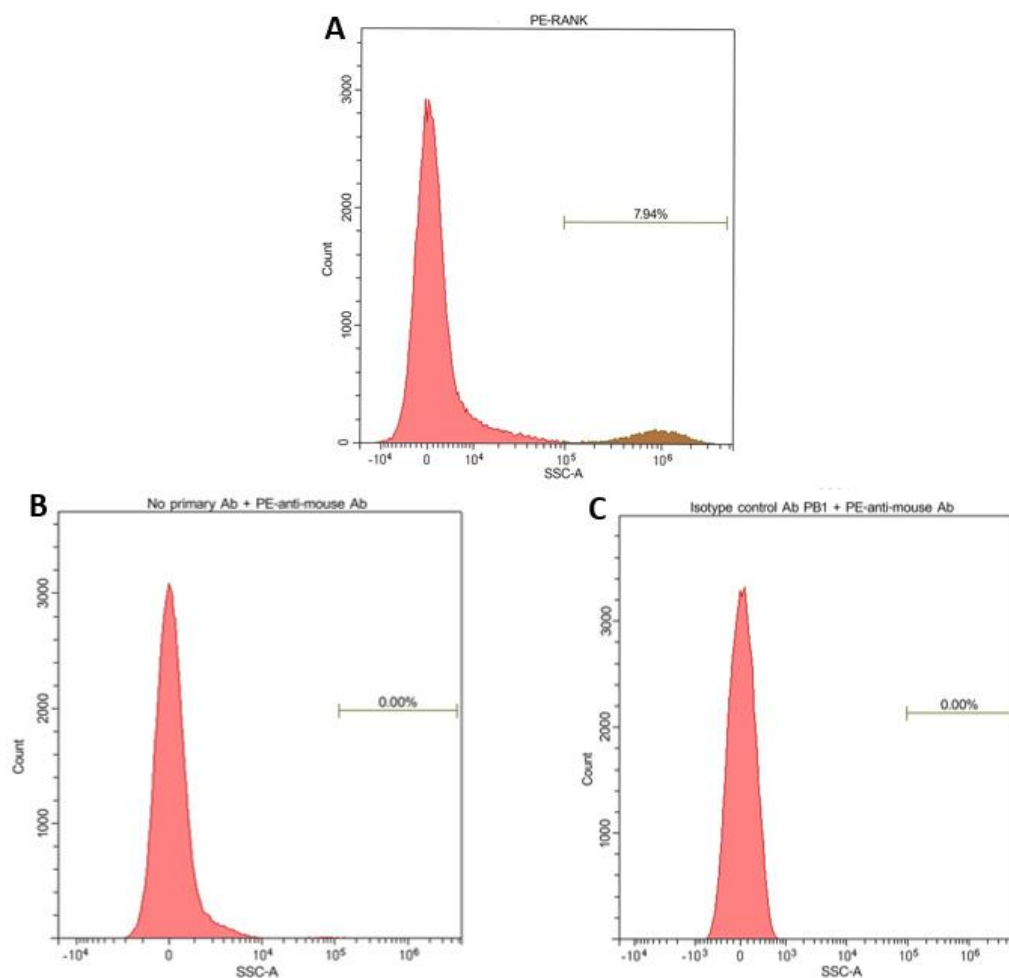


Figure 9 (A) A representative histogram showing the percentage of membranous vesicles positive for phycoerythrin (PE)-conjugated anti-human RANK antibody at 7.94. A representative histogram showing no immunoreactivity by replacing the PE-conjugated anti-human RANK antibody with PBS (B; no primary antibody) or with the isotype control antibody PB1 (C). SSC-A = side scatter area.

The osteogenic induction of periodontal ligament cells by osteoclast conditioned medium

Treatment of the PDL cells with OC-CM significantly upregulated *RUNX2* and *OSX* on day 1 compared with a negative untreated control ($p < 0.05$; Figure 10.1A, B). On day 7, there was significant upregulation of *OSX* and *BSP* compared with the negative untreated control ($p < 0.05$; Figure 10.1B) ($p < 0.01$; Figure 10.1F).

Treatment of the PDL cells with OC-CM significantly increased ALP staining and biomineralization as assessed by Alizarin red and von Kossa staining, compared with the negative untreated control ($p < 0.05$; Figure 10.2A-C).

Inhibition of RANK-RANKL signaling for osteogenic induction of osteoclast conditioned medium treated periodontal ligament cells

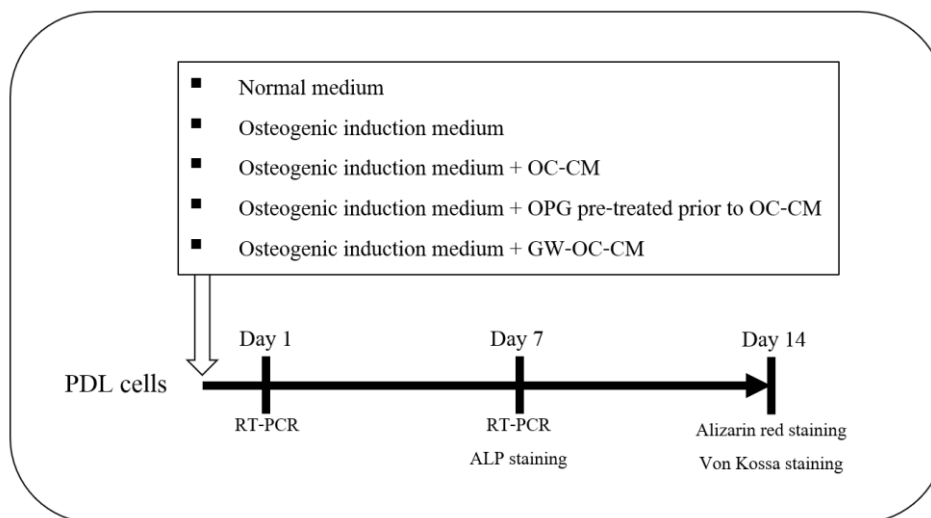
Pretreatment of the PDL cells with OPG prior to OC-CM significantly downregulated *RUNX2* and *OSX* on day 1 compared with OC-CM-treated PDL cells ($p < 0.05$; Figure 10.1A, B). On day 7, there was significant upregulation of *OSX* and *BSP* compared with OC-CM-treated PDL cells ($p < 0.05$; Figure 10.1B) ($p < 0.01$; Figure 10.1F).

Pretreatment of the PDL cells with OPG prior to OC-CM significantly decreased ALP staining and biomineralization as assessed by Alizarin red and von Kossa staining, compared with OC-CM-treated PDL cells ($p < 0.05$; Figure 10.2A-C).

Investigation of the role of extracellular vesicles in osteoclast conditioned medium on osteogenic induction of periodontal ligament cells

Treatment of the PDL cells with GW-OC-CM significantly downregulated *RUNX2* and *OSX* on day 1 compared with OC-CM treated PDL cells ($p < 0.05$; Figure 10.1A, B). On day 7, there was significant upregulation of *OSX* and *BSP* compared with OC-CM treated PDL cells ($p < 0.05$; Figure 10.1B) ($p < 0.01$; Figure 10.1F).

Pretreatment of the PDL cells with GW-OC-CM significantly decreased ALP staining and biomineralization by Alizarin red and von Kossa staining, compared with OC-CM treated PDL cells ($p < 0.05$; Figure 10.2A-C).



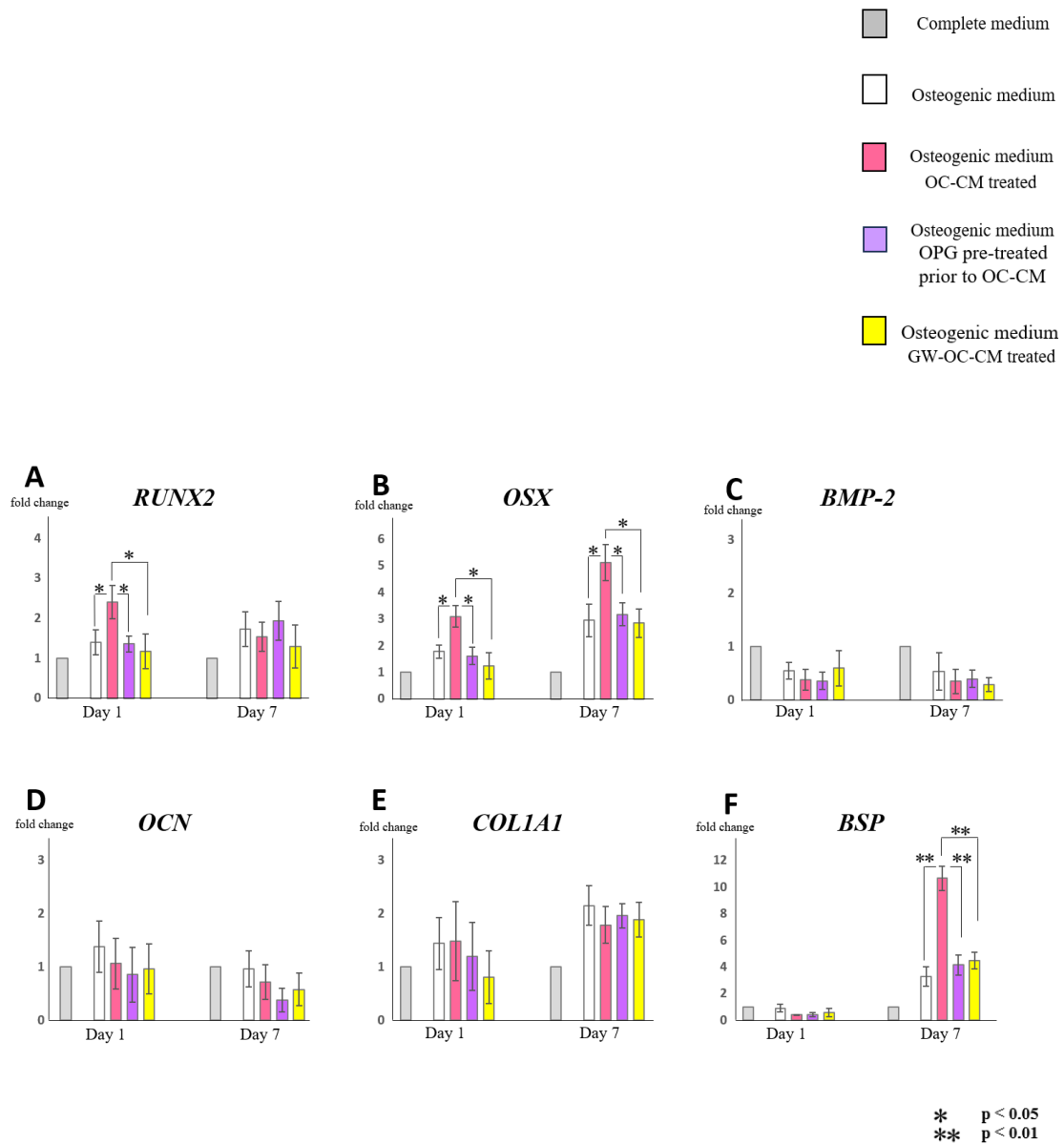


Figure 10.1 Osteogenic gene expressions at day 1 and 7 of periodontal ligament cells treated with OC-CM, GW-OC-CM, and pretreated with OPG prior to OC-CM.

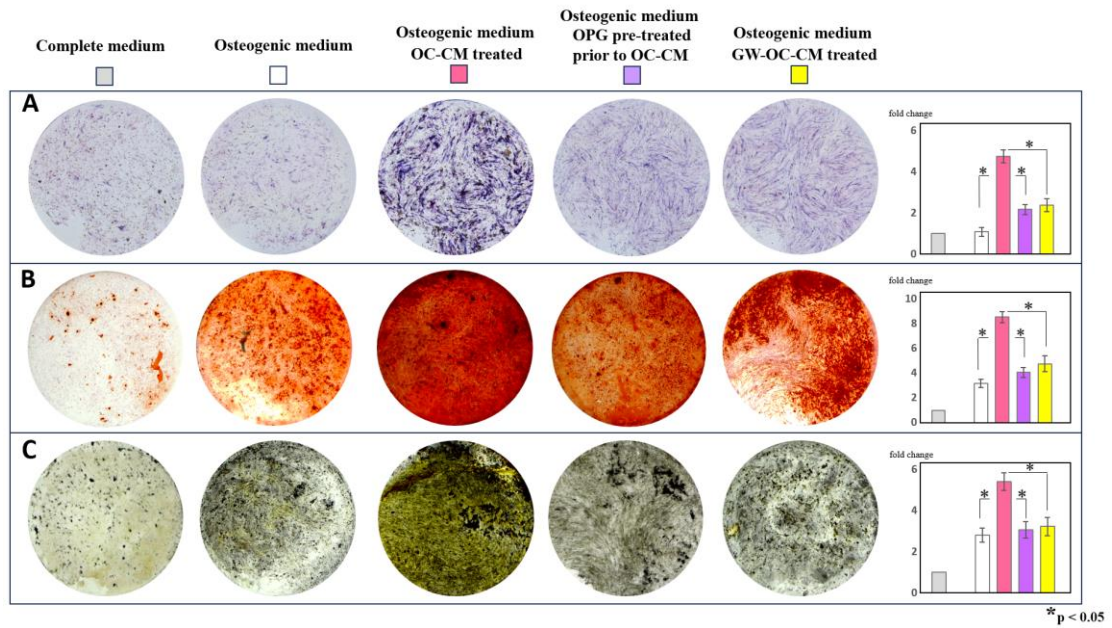


Figure 10.2 ALP staining (A) and biomineralization (B, C) of periodontal ligament cells treated with OC-CM, GW-OC-CM, and pretreated with OPG prior to OC-CM

The cytotoxic effects of DMSO and GW4869 on mature osteoclasts

0.005% DMSO and 20 μ M GW4869 had no cytotoxic effects on mature osteoclasts, as shown by similar percentages of viability in all treatments compared to the non-treated group (Figure 11).

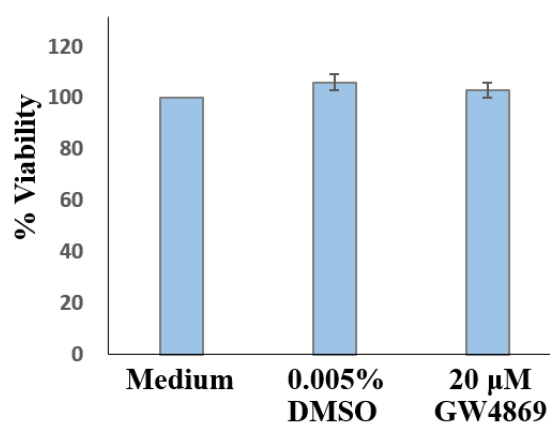


Figure 11 Cytotoxic effects of 0.005% DMSO and 20 μ M GW4869 on mature osteoclasts.

Co-localization of RANK and RANKL

PDL cells treated with OC-CM were incubated with PE-conjugated anti-RANK antibody, which showed co-localization with Alexa Fluor® 488-conjugated anti-RANKL antibody on the PDL cells (Figure 12B). No immunostaining with the anti-RANK antibody was observed on the cells treated with GW-OC-CM (Figure 12C), similar to the untreated PDL cells (Figure 12A)

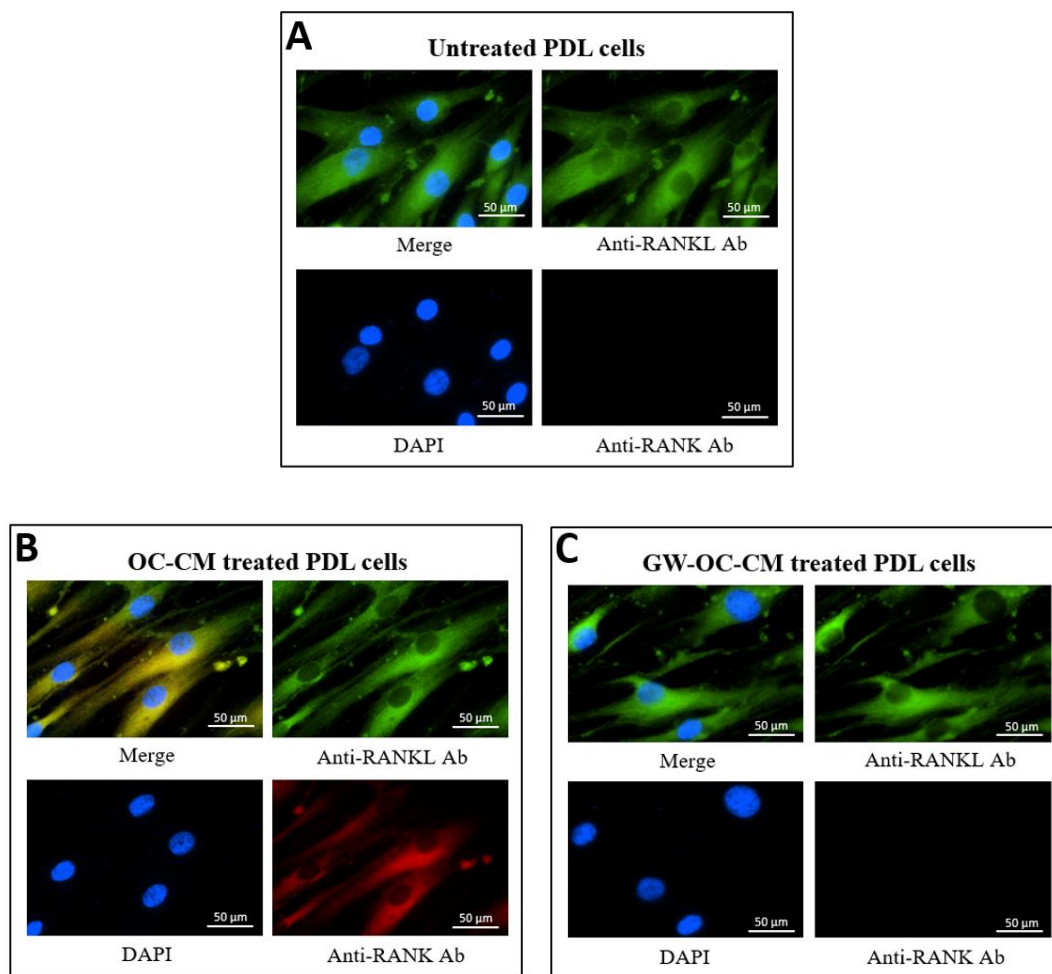
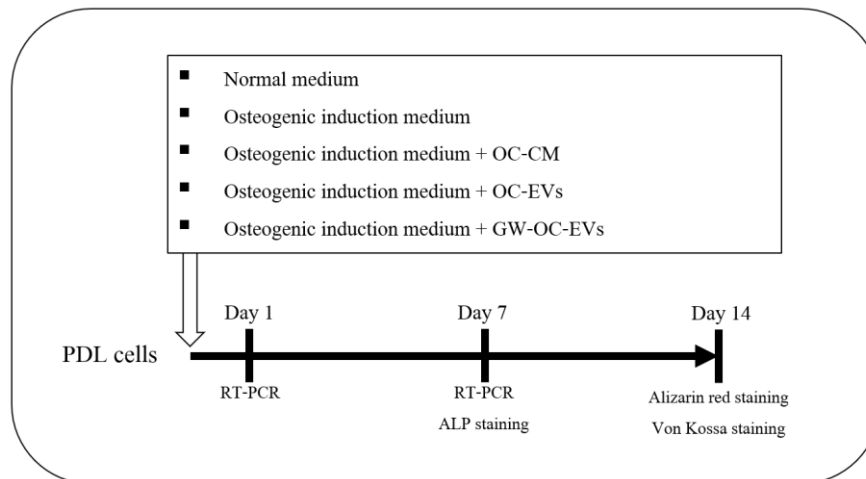


Figure 12 (A) The absence of red immunostaining with anti-human RANK antibody in periodontal ligament cells that were left untreated with OC-CM or treated with GW-OC-CM (C). PDL cells treated with OC-CM exhibited colocalization of RANK and RANKL (B).

Elucidation of the role of osteoclasts' extracellular vesicles on osteogenic differentiation of periodontal ligament cells



The PDL cells were treated with the isolated EVs at approximately $1,250 \times 10^6$ in 1 ml of culture medium (equivalent to OC-CM at a ratio of 1:10 (v/v) in culture medium). Treatment of the PDL cells with OC-EVs significantly upregulated *RUNX2* on day 1 and *OSX* on day 7, compared with a negative untreated control and GW-OC-EVs treated PDL cells ($p < 0.05$; Figure 13.1A, F). On day 7, BSP was significantly upregulated compared with a negative untreated control and GW-OC-EVs treated PDL cells ($p < 0.05$; Figure 13.1F). Similarly to OC-CM treatment, OC-EVs significantly increased ALP staining and biomineralization by Alizarin red and von Kossa staining, compared with the negative untreated control and GW-OC-EVs treated PDL cells ($p < 0.05$; Figure 13.2A-C).

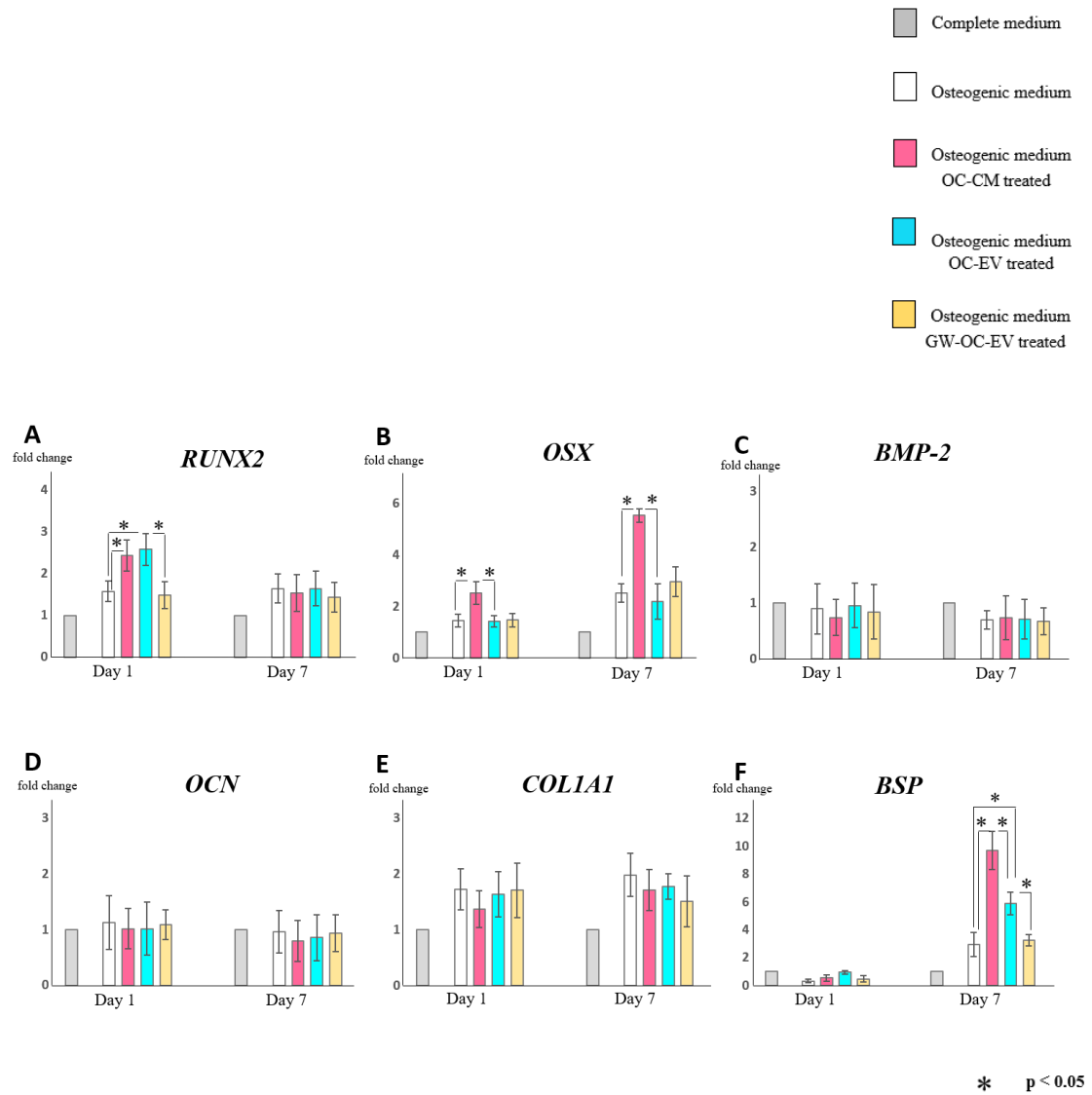


Figure 13.1 Osteogenic gene expressions on days 1 and 7 of periodontal ligament cells treated with OC-CM, OC-EVs, or GW-OC-EVs.

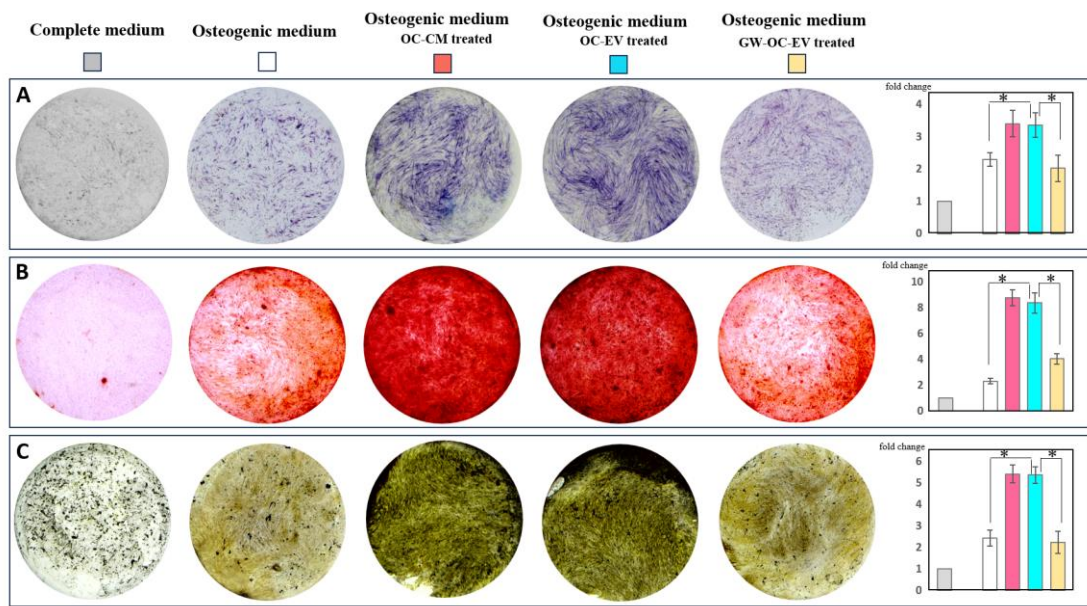
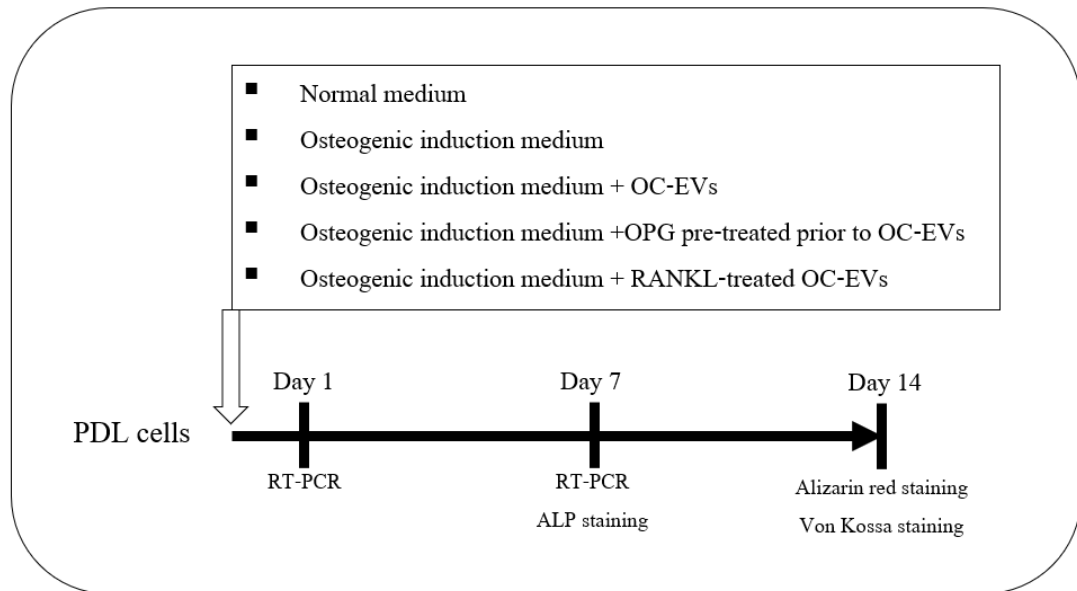


Figure 13.2 ALP staining (A) and biomineralization (B, C) of periodontal ligament cells treated with OC-CM, OC-EVs, or GW-OC-EVs.

Investigation of RANK - RANKL signaling for osteogenic induction of periodontal ligament cells treated with osteoclasts' extracellular vesicles



Upregulated mRNA expressions of *RUNX2* on day 1 (Figure 14.1A) and *BSP* on day 7 (Figure 14.1F) upon treatment of PDL cells with OC-EVs were significantly downregulated in OPG-pretreated PDL cells and PDL cells treated with RANKL-treated OC-EVs ($p < 0.05$), consistent with the suppressive effect on ALP staining (Figure 14.2A; $p < 0.05$) and biomineralization (Figure 14.1B, C; $p < 0.05$).

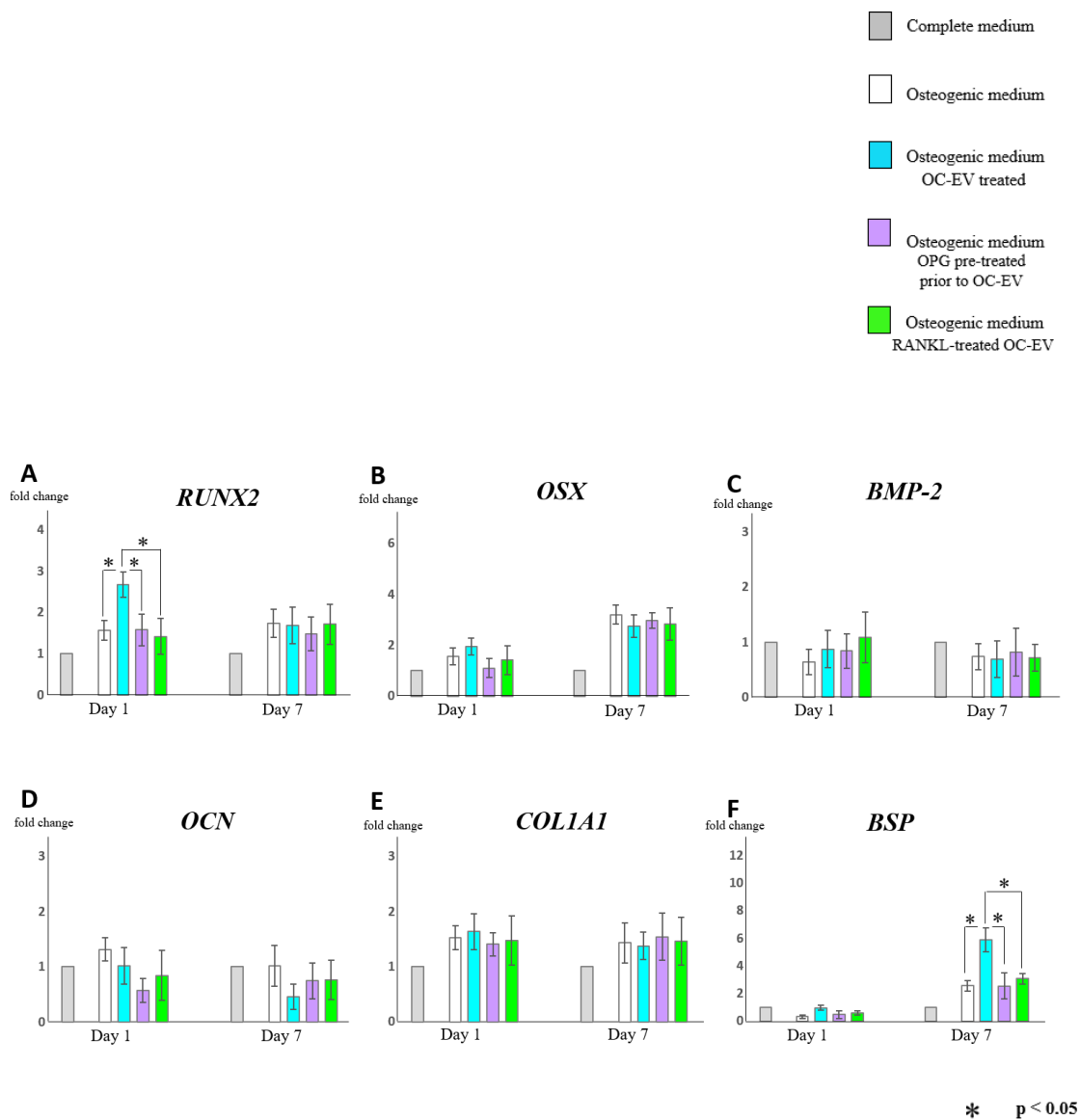


Figure 14.1 Osteogenic gene expressions on day 1 and 7 of periodontal ligament cells treated with OC-EVs, pretreated with OPG prior to OC-EVs, and RANKL treated OC-EVs.

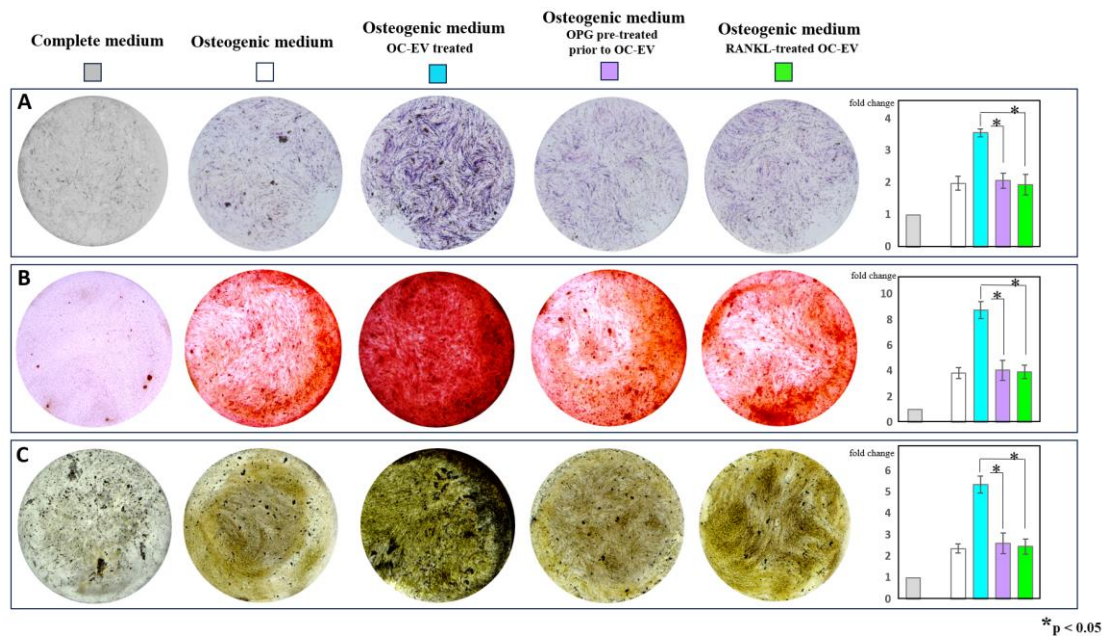
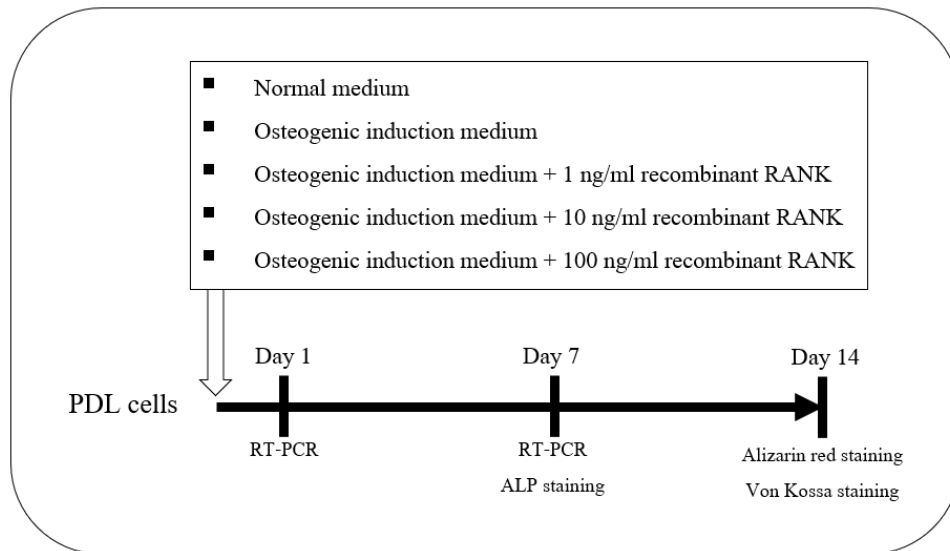


Figure 14.2 ALP staining (A) and biomineralization (B, C) of periodontal ligament cells treated with OC-EVs, pretreated with OPG prior to OC-EVs, and RANKL treated OC-EVs

Effect of recombinant RANK on the osteogenic differentiation and biomineralization of periodontal ligament cells



No significant difference in *RUNX2* (Figure 15.1A), *OSX* (Figure 15.1B), and *OCN* (Figure 15.1C) expressions on days 1 and 7 of PDL cell treatment with different doses of recombinant RANK.

For *COL1A1* expression, treatment of PDL cells with 1, 10, and 100 ng/ml recombinant RANK showed significant upregulation on day 7 ($p < 0.05$) (Figure 15.1D).

No significant difference in ALP staining (Figure 15.2A), Alizarin red staining (Figure 15.2B), and von Kossa staining (Figure 15.2C) of PDL cell treated with different doses of recombinant RANK compared with the osteo-induction medium group.

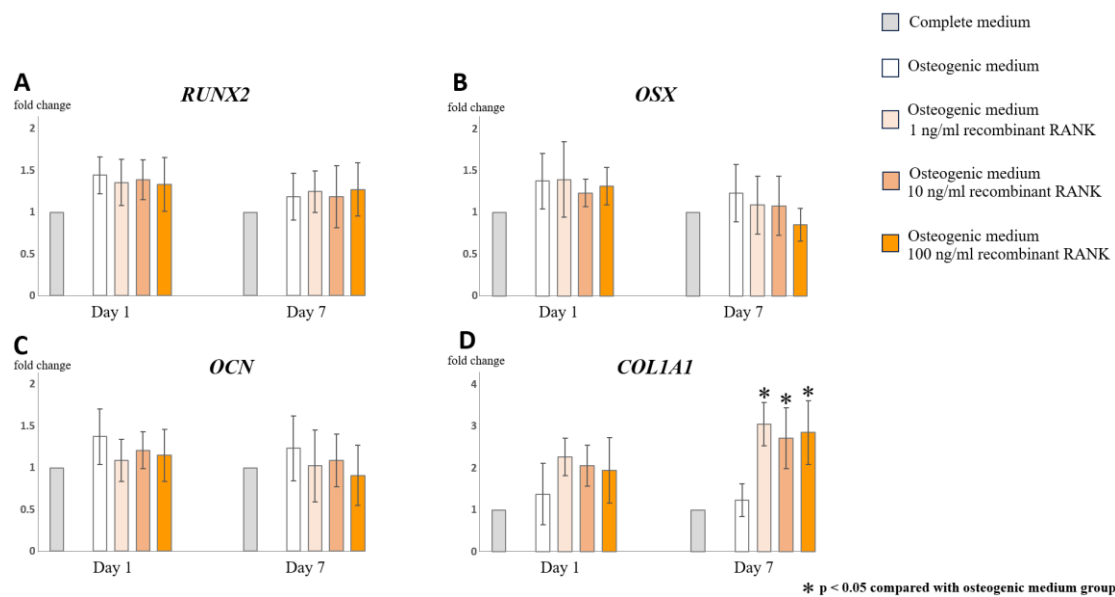


Figure 15.1 Osteogenic gene expressions on days 1 and 7 of recombinant RANK treated periodontal ligament cells.

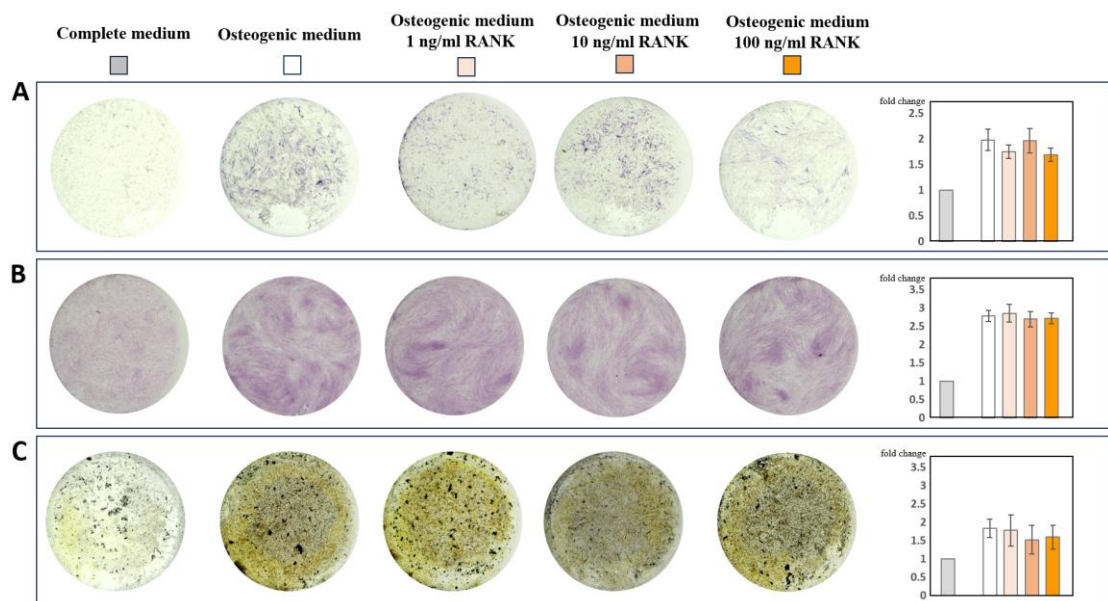


Figure 15.2 ALP staining (A) and biomineralization (B, C) of recombinant RANK treated periodontal ligament cells

CHAPTER 5

DISCUSSION AND CONCLUSION

In this study, the RANK-RANKL reverse signaling from mature human osteoclasts to induce osteogenesis of human PDL cells was demonstrated *in vitro*. This study is consistent with previous studies that investigated the induction of mesenchymal cells to upregulate osteogenic genes and undergo biomineralization through the RANK-RANKL signaling pathway.^(3, 61, 93, 114) By using of recombinant OPG to treat cells before treating them with OC-CM or OC-EVs. which help confirm the role of the RANK-RANKL reverse signaling pathway in osteogenesis.^(3, 114) In addition to using recombinant RANKL for the antagonist of RANK on EVs.⁽³⁾ These conformities suggest that the role of RANK-RANKL reverse signaling pathway in osteogenesis can be separately tested on either end of this pathway.

A previously established paradigm of cell-to-cell interaction between PDL cells and osteoclasts is a one-way direction from RANKL of PDL cells to RANK of osteoclasts to drive osteoclast formation and activation for bone catabolism.^(45, 115, 116) A recent study in 2022 by Xian-Min Liao *et al* demonstrated that human PDL cells were induced to undergo osteogenic differentiation by EVs from macrophages through miRNAs.⁽⁵⁴⁾ However, the reverse effect of mature osteoclasts on osteogenic differentiation of PDL cells was demonstrated for the first time, which could provide new insights into the role of osteoclasts in alveolar bone regeneration. Nevertheless, this reverse effect is not likely to play a major role in bone regeneration, since various secreted pro-inflammatory cytokines from pathological conditions to promote

osteoclastogenesis often override the reverse effect.⁽¹¹⁷⁾ Biological changes during orthodontic tooth movement are generally known but remain unclear in many aspects. The Biphasic Theory of Orthodontic Tooth Movement, a new paradigm of tooth movement, states that osteoclasts are main players in an initial catabolic phase, followed by an anabolic phase.⁽¹⁶⁾ Transition from the catabolic to anabolic phase is not clearly demarcated, which is evident by the presence of osteoclasts at both compression and tension sides, shown in histological sections of mouse molars undergoing force application.⁽¹¹⁸⁾ Currently, there is no specific explanation for the appearance of osteoclasts at the tension side. It is possible that the reverse effect of mature osteoclasts on osteogenesis of PDL cells may help explain this unexplained phenomenon. It is likely that the presence of osteoclasts at the tension side may be required to promote osteogenesis of both bone stromal and PDL cells.

In this study, the isolated EVs were characterized for morphology using TEM, which showed particles with a bilayer membrane. Flow cytometry using a membrane labeling dye (ACO-600) revealed that 59.31% of the total particles were lipid bilayer membranous particles (Figure 4). However, when stained for RANK and analyzed by flow cytometry, only 7.94% of the particles were positive for RANK (Figure 9A). For further studies, OC-EVs should be stained for RANK in TEM.

EVs isolated through ultracentrifugation are often contaminated with non-vesicular molecules, which pose a challenge for functional analysis.^(119, 120) To address this issue, the present study employed an additional purification step using a 0.22- μ m filter to eliminate apoptotic bodies, microvesicles, and cell debris during the isolation process. The NTA results show that the size of the particles in the isolated EVs does not vary significantly. The largest particle size in OC-EVs was 256 nm, with a very low

quantity (less than 0.1 particles/ml), while the largest particle size in GW-OC-EVs was 379 nm, with an approximate quantity of 0.5 particles/ml. Although it is unclear what these particles are, the experimental results indicate that GW-OC-EVs did not show significant differences in gene expression and biomineralization compared to the control group. However, it cannot yet be concluded whether the particles around 379 nm in GW-OC-EVs could potentially affect the experimental outcomes. Furthermore, it remains unclear whether the treatment of osteoclasts with GW4869 affects the release of particles within the size range of microvesicles and small apoptotic bodies, and further research is needed to investigate this.

The identification of the EV characteristics of the isolated particles in this study was performed through Western blot analysis to detect EV-specific protein markers, as well as to analyze proteins that should not be present in EVs, such as Lamin A/C (which is found in the nucleus) and Calnexin (which is found in the endoplasmic reticulum). The Western blot results, when compared to the protein levels in the cell lysate of mature osteoclasts, clearly indicated EV protein markers. However, the intensity of the bands corresponding to EV proteins was significantly lower than those of cellular proteins. When considering GAPDH, it was also found to be much less intense in the EVs. Although the total protein concentration was measured before the Western blot to ensure equal loading of proteins from both the cells and EVs, these findings suggest that OC-EVs contain a considerable amount of non-EV proteins, which is consistent with the flow cytometry data presented in Figure 4.

GAPDH has long been considered a housekeeping gene and protein due to its essential role in glycolysis and its presumed stable expression across various cell types and conditions. Recent studies have revealed that GAPDH possesses multiple roles in

cellular processes. GAPDH is not only involved in glycolysis but also plays a role in regulating transcription and initiating apoptosis. It can move between the cytoplasm and the nucleus, connecting the cell's metabolic state to gene expression. Furthermore, under oxidative stress, GAPDH can trigger cell death by binding to DNA and interacting with other proteins.⁽¹²¹⁾ GAPDH also participates in vesicle transport, particularly in moving proteins from the endoplasmic reticulum to the Golgi apparatus, which is crucial for protein secretion. It is recruited by Rab2 to vesicular-tubular clusters and aids in the formation of vesicles.⁽¹²²⁾ GAPDH has been found to assist in maintaining iron balance within cells by acting as a chaperone for labile heme, which plays a role in iron homeostasis.⁽¹²³⁾ Due to its involvement in various cellular functions, GAPDH expression can fluctuate depending on experimental conditions, cell types, or stress responses. This variability could affect its reliability as a housekeeping gene or protein for normalization in experiments, such as PCR or Western blotting. Therefore, it is important to validate GAPDH's stability under the specific conditions of the study before using it as a reference.

In a study investigating the co-localization of two proteins in cells through immunofluorescence staining with two dyes, it is important to consider whether their emission wavelengths overlap. PE and Alexa Fluor® 488 used in this study emit light in the ranges of 578-610 nm and 500-550 nm, respectively. If the emission spectra of the two dyes overlap, it could interfere with the co-localization analysis when using a fluorescence microscope, especially if both signals are detected within the same channel. This phenomenon is known as cross-talk, where the fluorescence of one dye could be detected in the channel intended for the other, leading to erroneous co-localization results. While there is some overlap in the emission spectra of PE and Alexa

Fluor® 488, careful experimental design and appropriate filtering can reduce this overlap, ensuring reliable co-localization results in fluorescence microscopy. ⁽¹²⁴⁾

GW4869, an inhibitor of neutral sphingomyelinase, which functions as an enzyme involved in the external transport of EVs, was used to treat mature osteoclasts prior to the collection of OC-CM to inhibit the osteogenic induction of RANK-containing EVs in the conditioned medium of mature human osteoclasts. The use of GW4869 to study the role of EVs within CM in this research is consistent with several previous studies. ^(3, 6, 56) Observation under a microscope revealed the entrapment of vesicles within osteoclasts treated with GW4869 during culture, as shown in Figure 16. Additionally, this study confirmed the inhibitory effect of GW4869 on EV release by comparing particle quantities using NTA. It was found that GW-OC-EVs contained 93.8 million particles/ml, while OC-EVs contained 12.5 billion particles/ml. Moreover, immunofluorescence for co-localization of RANK and RANKL on PDL cells was negative (Figure 12). Based on several experimental results, it can be suggested that GW4869 inhibits the release of EVs from osteoclasts, preventing the RANK-RANKL signaling pathway, which subsequently leads to reduced osteogenic differentiation of PDL cells.

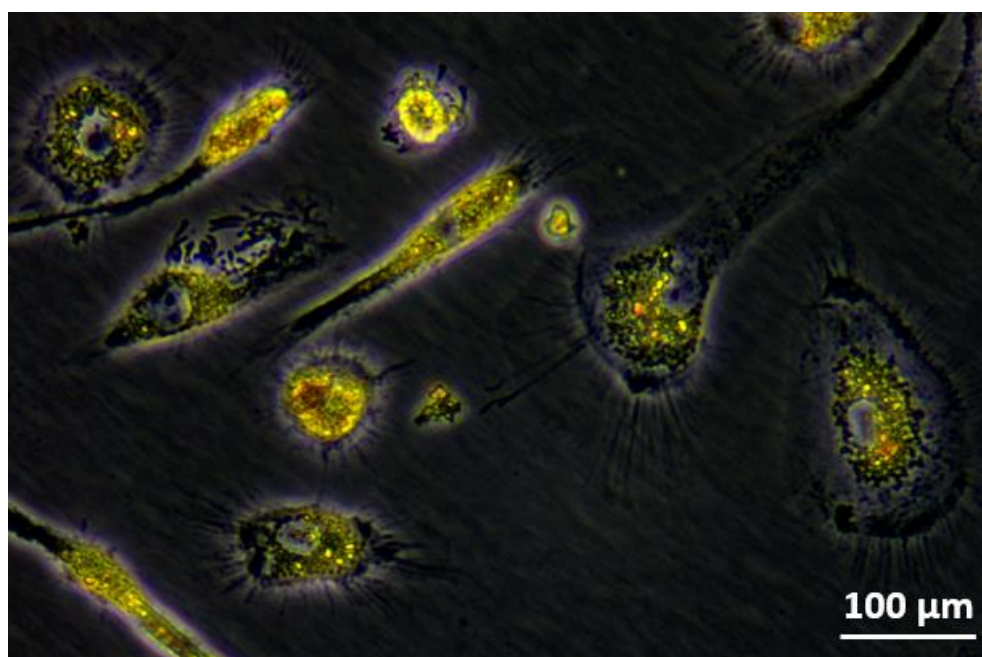


Figure 16 Entrapment of vesicles within mature osteoclasts after treatment with 20 μM of GW4869 for 3 days

As shown in Figures 15.1 and 15.2, recombinant RANK was unable to enhance osteogenic differentiation and biomineralization of PDL cells in this study. This contrasts with a previous study by Zhang et al,⁽⁶¹⁾ who found that recombinant mouse soluble RANK can stimulate osteogenic differentiation and biomineralization in osteoblast cell lines, with an increase in *OCN* observed between days 6 and 18. On the other hand, this study only observed upregulation of *COL1A1* on day 7 when PDL cells were treated with recombinant RANK, but no changes in the *OCN* gene were found (Figure 15.1), nor was there any enhancement of biomineralization (Figure 15.2). The discrepancy in experimental results may be explained by the need for suitable linker peptide sequences and orientations of the RANK protein, which are essential for specific fusion with the RANKL proteins on the surface of PDL cells. A study has attempted to predict the binding pattern complexity between RANK and RANKL in order to optimize their use for pharmacological applications.⁽³⁾ However, the exact mechanism of RANKL binding with RANK remains elusive. There is no structural information available for the RANK/RANKL complex, although the crystal structure of the extracellular domain of RANKL has been obtained and resolved.⁽¹²⁵⁾ The increase in *COL1A1* in this study may suggest that recombinant RANK can bind to RANKL on the surface of PDL cells to some extent. However, the binding may not be strong or stable enough to trigger significant signaling events in the cells that are critical for osteogenic differentiation.

Of the six osteogenic genes studied in the OC-CM and OC-EVs experiment, it was found that three genes (including *RUNX2*, *OSX*, and *BSP*) exhibited significant changes in expression levels. The expressions of both *RUNX2* and *OSX* are considered central regulators of the early stages of osteogenic differentiation. *OSX* is known to be

involved in osteoblast maturation and activity in addition to early osteoblast differentiation.⁽¹²⁶⁾ *OSX* also functions as a late marker of osteoblast differentiation and regulates osteogenesis in multipotent human mesenchymal stromal cells and PDL cells.⁽¹²⁷⁾ Consequently, the significantly upregulated *RUNX2* mRNA expression in human PDL cells treated with OC-CM was observed only on day 1, whereas *OSX* mRNA upregulation was observed on both days 1 and 7. For treatment with OC-EVs, only significantly upregulated *RUNX2* mRNA expression was found only on day 1.

Upregulation of *BSP* mRNA expression in human PDL cells on day 7 was consistent with the results of previous studies.^(113, 128) *BSP*, a major non-collagenous protein of bone, plays a key role in de novo bone formation by controlling mineralization in the late stage of osteogenesis.⁽¹²⁹⁾

OCN, *COL1A1*, and *BMP-2* are the three genes that did not show significant changes in expression in PDL cells treated with OC-CM or OC-EVs. However, several previous studies have observed changes. For instance, *BMP-2* was found to be elevated when human MSCs were treated with EVs or CM from human osteoclast lineage.^(63, 64, 67) *COL1A1* levels increased in mouse MSCs when treated with CM or EVs from osteoclasts.^(3, 56) Additionally, *OCN* expression was found to be upregulated in human PDL cells when treated with EVs from macrophages.⁽⁵⁴⁾

One previous study,⁽⁵⁶⁾ which compared gene expression levels and biomineralization between treatments of mouse MSCs with OC-CM and OC-EVs, found that the expression levels of the genes *Runx2*, *Osx*, and *Col1a1*, as well as biomineralization, were higher in both OC-CM and OC-EVs compared to the control group, with no significant difference between OC-CM and OC-EVs. This study found

a difference in the gene expression levels between treatment with OC-CM and OC-EVs. It was observed that treatment with OC-EVs did not increase the level of *OSX* as OC-CM did, both on day 1 and day 7. In the case of *BSP*, although treatment with OC-EVs increased gene expression levels compared to the control group, they remained significantly lower than those in the OC-CM group. However, no significant difference was found in the levels of ALP staining and biomineralization in this study.

It is possible that the CM contains signaling molecules that can induce the upregulation of osteogenic genes. Fernandes *et al*⁽⁶⁴⁾ reported that Oncostatin M (OSM) in OC-CM can induce osteogenic differentiation of MSCs. OSM, a multifunctional cytokine of the interleukin-6 family, regulates osteogenic differentiation of MSCs from several sources.⁽¹³⁰⁻¹³⁵⁾ In addition, OSM enhances the hematopoietic supportive activity of bone marrow stromal cell lines. OSM potently stimulates RANKL expression in osteoblasts which may lead to osteoclastogenesis.^(131, 132, 136) In this study, it is expected that OSM may be present in the OC-CM and have a greater effect on upregulating osteogenic genes than isolated EVs.

Besides OSM, it has been reported that osteogenic differentiation of mouse MSCs is induced by glial cell-derived neurotrophic factor (GDNF) present in the conditioned medium obtained from osteoclasts.⁽⁵⁸⁾ GDNF is a crucial growth factor that plays a vital role in the proliferation, migration, and differentiation of nerve cells. It also promotes the bone regenerative abilities of MSCs by activating the PI3K/Akt signaling pathway.⁽¹³⁷⁾

Additionally, although this study aimed to equalize the number of EVs from OC-CM and OC-EVs (1,250 million particles/ml) by adjusting the volume of PBS used

for resuspension to match the amount of CM used to isolate the EVs, there is often a loss of EVs due to the ultracentrifugation and filtration processes. ^(138, 139)

In addition to the RANK-RANKL signaling observed in this experiment, it is possible that other signaling pathways are involved and contribute to inducing osteogenic differentiation through EVs.

The ephrin/Eph signaling system mediates cell–cell contact signaling and the process is bidirectional. Soluble ephrins, by themselves, cannot activate Eph receptor signaling unless they are either attached to the membrane or artificially aggregated. ⁽¹⁴⁰⁾ It has been reported that cancer cells release Ephrin-B containing EVs. ⁽¹⁴¹⁾ The cell-surface molecule of osteoblasts, EphB4, and EphrinB2, as well as the transmembrane protein of osteoclasts, have been reported by several studies to play a role in regulating bone metabolism through a bidirectional signaling mechanism. ^(38, 39, 142) EphB4-ephrinB2 reverse signaling suppresses osteoclast formation, while ephrinB2-EphB4 forward signaling enhances osteoblast formation. ⁽³⁸⁾ In beagle dogs' periodontal ligament cells, it has been studied that osteogenic differentiation can occur through ephrinB2-EphB4 signaling. ⁽³⁹⁾

As studied by Liao *et al* ⁽⁵⁴⁾ regarding the osteogenic differentiation of PDL cells, various miRNAs within EVs from macrophages play a role in inducing osteogenic differentiation. Therefore, it is possible that miRNAs are involved in the OC-EVs induced osteogenic differentiation in this study, which may also include miRNAs that play an inhibitory role. However, the conditions that determine when and which miRNAs will perform specific functions are still an area that requires further

investigation. However, The heterogeneity of EVs does not occur randomly but is clearly regulated to align with the type of recipient cells, which are also clearly defined targets. For example, in the study by Li et al ⁽⁴⁾ on osteoclast-derived exosomal miR-214-3p inhibiting osteoblastic bone formation, the experiment involved gene mutations to increase or decrease miRNA levels. In reality, it is believed that there is an equilibrium of regulatory factors in exosomes. Under normal conditions, OC-EVs tend to stimulate bone formation, but in patients with pathologies such as bone metabolic diseases, this equilibrium of regulatory factors is altered in a way that corresponds with the disease. ^(4, 90) Additionally, from several experiments studying EVs from osteoclast lineages, it was found that there are certain mechanisms that inhibit the osteogenic differentiation of mesenchymal cells as well, as shown in Table 3.

Table 3 List of studies on the regulation of mesenchymal stem cell lineages by osteogenic inhibition or bone resorption enhancement via conditioned medium or extracellular vesicles from the osteoclast lineage.

Study	Source of CM or EVs	Target cells	Signaling molecule/pathway
Huyan 2019 ⁽⁸⁹⁾	osteoclasts (RAW264.7) EVs	MC3T3-E1	Microgravity via Wnt/ β -catenin
Gebraad 2018 ⁽¹⁴³⁾	LPS induced-monocytes & osteoclasts (primary human cells) EVs	adipose tissue- derived MSCs & bone stromal cells)	MMPs stimulation

Li 2016 ⁽⁴⁾	osteoclasts (primary human cells) (RAW 264.7) EVs	osteoclasts (human cell line) (primary mouse cells)	miR-214-3p
Huynh 2016 ⁽⁹³⁾	osteoclasts (primary mouse cells) EVs	mouse MSCs	RANK-RANKL
Sun 2016 (30)	osteoclasts (RAW 264.7) EVs	MC3T3-E1	ephrinA2/EphA2 miR-214/ATF4
Yang 2020 (6)	osteoclasts (RAW 264.7) OC-EVs, GW-OC-EVs	MC3T3-E1	miR-23a-5p

Overall, the results of this study have led to new insights regarding cells in the alveolar bone. Previously, the understanding of the interactions between osteoclasts and PDL cells was limited to mechanisms of osteoclastogenesis and bone resorption. However, discovering an additional dimension of the relationship between these two cell types will serve as the foundation for future research to explain various phenomena of the alveolar bone that are still unclear. Moreover, this could lead to new knowledge about EVs, which are considered biological nanoparticles, potentially advancing drug delivery techniques for clinical interventions.

In summary, the osteogenic differentiation and biomineralization of human PDL cells can be enhanced by extracellular vesicles released from mature osteoclasts via RANK-RANKL reverse signaling.

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List of Publication and Proceedings :

1. Vibratory stimulation increases interleukin-1 beta secretion during orthodontic tooth movement. Leethanakul C, Suamphan S, Jitpukdeebodintra S, Thongudomporn U, Charoemratrote C. *Angle Orthod* 2016 Jan;86(1):74-80. doi: 10.2319/111914-830.1.
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